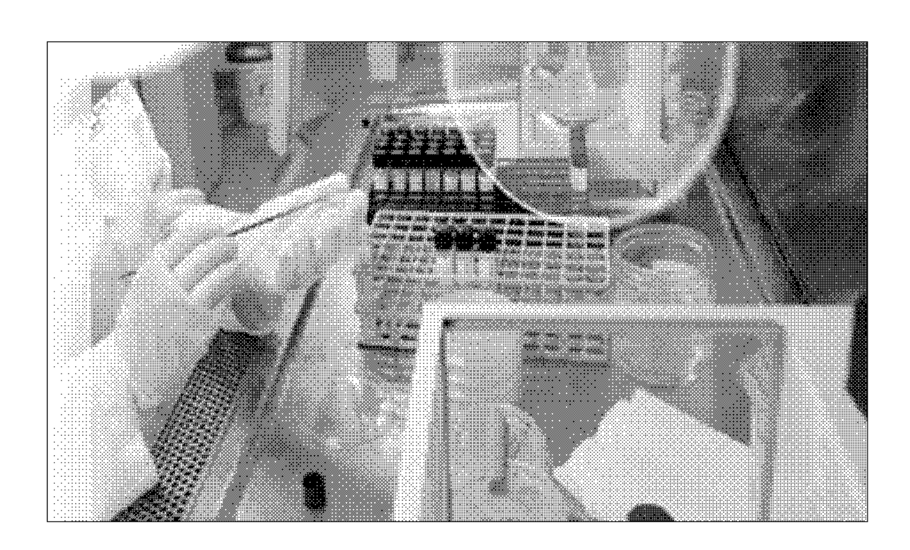
LECTURES ON APPLIED CLINICAL MICROBIOLOGY

By

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Introduction

Completely updated and in full color, book covers the base lines of diagnostic microbiology. A logical building-block approach supplies what students need to know in an easy-to-use, memorable format. Material is presented in a progressive manner, from basic principles and concepts to systematic identification of etiologic agents of infectious diseases, promoting greater understanding and the development of laboratory skills. The first chapter of the book explains basic principles and concepts, setting up a firm foundation of diagnostic microbiology. It summarizes the role of clinical microbiologists and technical jobs of junior and senior staff. It gives a good idea how to collect good microbiological samples for laboratory diagnosis. Building on these basics, Part II highlights simple culture methods for the identification of significant isolates. Further discussion will be carried out for clinical microbiology approach for diagnosis of organ systems infections with detailed discussion of the laboratory diagnosis of infectious diseases with a focus on the most medically significant and commonly encountered diseases.

Laboratory diagnosis of infectious diseases include bacterial pathogens such as Enterobacteriaceae -- Nonfermentative gram-negative bacilli -- Curved gram-negative bacilli and oxidase-positive fermenters: campylobacters and vibrionaceae -- Haemophilus -- Miscellaneous fastidious gram-negative bacilli -- Neisseria species and Moraxella catarrhalis -- Staphylococci and related organisms -- Streptococci and Streptococcus-like bacteria -- Aerobic gram-positive bacilli -- Anaerobic bacteria -- Antimicrobial susceptibility testing -- Mycobacteria -- Spirochetal infections -- Mycoplasmas and ureaplasmas -- Mycology -- Virology -- Conventional and new and technologies for rapid organisms identification and detection methods. There is also a simple approach for biosafety and biohazard in clinical microbiology laboratories and quality control procedures.

The Use of the Clinical Microbiology Laboratory (General Principles)

*** Collection of Clinically Relevant Specimens:**

The best use of the laboratory involves sending only relevant specimens so that work can be done on a reasonable no. of specimens especially when economic situation of the country is greatly limited

Examples of unnecessary investigations include routine microscopy and culture of urine specimens from all non catheterized adult patients in hospital without symptoms suggestive of urinary tract infection.

The role of the consultant clinical microbiologist:

A- Regular contact with clinical colleagues to ensure the appropriate investigation for the clinical conditions, and that good quality specimeas are sent

Organize the laboratory so that the relevant investigations are carried out reliably, safely and economically. Important results are communicated to colleagues and discussed.

B- As regard consultation on the investigation and management of patients with infection problems. Seeing patient on the words, temp. charts, drug shets. Etc., together with clinical colleagues

Discussing difficult clinical problems and management of out breaks of infectious disease

C-As regards Control of hospital infection: design implementation of policies on the use of antibiotics, isolation procedures, sterilization and disinfection

D- As regard teaching research: Education of medical staff about infections, the use of antibiotics, disinfectants. Etc.

-Research on epidemiology, diagnosis, treatment or prevention of infections.

Provision of essential clinical information:

The information routinely required in laboratory request: age, brief details of the main clinical condition , date of onset of the illness, antibiotic therapy, history of recent travel abroad, suspected source of infection.

Prior Discussion with microbiologist.

To achieve the best use of the laboratory certain types of investigation need to be discussed with the microbiologist. These include, assay of antibiotics the isolation of viruses, molecular biology tests, and the investigations of possible cross infection incidents

Proper Sampling in Clinical Microbiology Laboratory

Collection of good quality specimens:

Depends on

1- The optimal time of specimen collection.
2- The correct type of specimen
3- Well collected specimens with minimum contamination from normal flora of the patient or the person collecting the specimen.
4- Adequate amounts of each specimens and appropriate no. of specimens
5- Clearly labeled safe specimens

1- Optimal time of collection of collection of specimens:

-specimens for the culture of bacteria collected before the start of antibiotic therapy

-Blood cultures and blood films for malarial parasites are best collected just as the patient's temp starts to rise, however, when infective endocarditis is suspected, three blood culture sets collected with 24 hour irrespective of patient temp.
- Specimens for virus isolation are most likely to give positive results when collected during the most acute stages of the disease
-Serology is satisfactory when four fold or greater rising antibody titer is demonstrated in pained sera.
The 1st serum sample as early as possible in the disease course. Second in the convalescent stage
2-Correct types of specimens:
Examples:
Bacterial meningitis-------blood cultures CSF culture
Suspected gonorrhea -------cervical, urethral and rectal swab should be collected rather than high regional swabs.
3-Well collected specimens with minimum contamination from the normal flora:
Poor quality specimens include saliva instead of sputum or a salivary – mucoid sputum sample instead of a muco purulent sputum.
-Mid stream urine need careful collection to a void excess contamination by genital flora.
-A throat swab should not touch the buccal mucosa and the tongue depressed by a spatula.
-Vaginal speculum should not be wet with antiseptic solution during collection of high vaginal swab with care not to touch the lower vagina or perineum.
-Strict septic and antiseptic techniques are used for blood and CSF cultures to avoid contamination from skin flora or from the doctor.
4-Adeqate amounts of appropriate number of specimens:
The volume of blood for culture from adult
-5-10 ml per bottle and in children and neonates 1-5ml per bottle.
-Collection of early morning sputum specimens, and collection of adequate amount of early morning urine specimen for 3 successive days is required for the isolation of M.T.B.
-Patients with diarrhea ---at least 2 specimens of faeces is collected for culture of Salmonellae or Shigella.
-Serological investigations usually require paired sera.
5-Clearly labeled and safe specimens:
Specimens for microbiological investigations should be placed in leak – proof containers, and each container should be enclosed in plastic bag.
The hazards to staff handling leaking container s include acquiring enteric infection from feces, T.B. from sputum of an open case of pulm. T.B. and viruses s such as HCV, HBV, HIV, from leaking blood.

Transport of specimens to the laboratory

Many pathogenic organisms don't survive for long in clinical specimens kept at room temp. Examples include *Gonococci*, *Haemophilus*, Bacteroides, anaerobic cocci and most viruses.

On the other hand, some organisms contaminating specimens from the normal flora such as Coliform and Coagulase negative *Staphylococci*, may rapidly grow in specimen kept at room temp.

-Urine or sputum specimens should reach the laboratory within 2hours of collection when even possible. If delay are expected immediately inoculated into transport media.

-Transport media used:

Stuart's transport media ----- for pus or swabs for bacterial culture when delays in transport.>1/2hour or when *Neisseria* infections are suspected. However the inoculated transport media should be sent to the laboratory within 4h.

The investigation of eye, genital tract is best carried at the bed side when suitable culture media are directly inoculated.

-Cerebrospinal fluid(CSF) not refrigerated since other wise *Meningococci* may rapidly die.

-Viral transport media is necessary for virus isolation, and also for Chlamydia isolation. Specimens for virus isolation are kept at –70°C till time of transferring the appropriate cell line which support growth of the possible virus or Chlamydia.

Basic Laboratory procedures for microbiological diagnosis

Laboratory procedures for microbiological diagnosis Include the following steps:

-Naked eye examination of Specimens

-Microscopy.

-Detection of microbial antigens.

-Isolation of microbes

- Antibiotic sensitivity

-Serology.

-Molecular biology techniques.

-Gas – liquid chromatographic techniques.

-Skin tests.

I. Naked eye examination of specimens:-

This helps to determine whether a specimen is suitable or no.

- A saliva sample instead of an expectorated sputum sample should be discarded.

-Turbid CSF, is an immediate evidence of infection

-A foul smelling pus specimen may suggest presence of anaerabes.

-A rice water stool sample may indicate *Vibrio cholera* infection

-Anchory sauce sputum sample would suggest invasive Amoebiasis in lungs.

-Sulphar granules in pus would indicate Actinomycosis.

II. Microscopy:

1-Wet preparation for light microscopy in examination of CSF, urine, body fluid for evidence of pus cells, and organisms.

-Vaginal secretion-- *Trichomonas* and *Candida* .

-Skin, nail , hair (in KaOH) -evidence of fungus
-Dark ground illumination to look for Spirochates, Treponema pallidum in suspected 1ry or 2ry .
2-Gram stained smear: It may help of saving life.
-Important in rapid diagnosis of bacterial meningitis on exam of CSF deposit.
-Diagnosis of Strepto pneumonia in sputum smear
-Performed in serious septicemia when a blood culture bottle is flagged positive.
- Identification of colonies appearing on culture media
-Gram – stained smear may give positive results al though the subsequent cultures are negative as a result of given antibiotics
-In Vincent's angina: stained smear the only means of diagnosis
3-Acid fast stain of sputum allow rapid diagnosis of open pulmonary tuberculosis demonstrating acid fast bacilli. In other, clinical specimens such as urine, peritoneal fluid, CSF it lacks sensitivity.
4-Immunoflourescent microscopy is important for rapid diagnosis of viral infection e.g. Respiratory syncytial virus (RSV) in infants and children, Herpes simplex, cytomegalovirus (CMV) in urine throat swab, Rabies in brain biopsy specimen, Clamydia trachomatis in conjunctival scrapings. Also, it can be used in serological antibody test e.g Fluorescent Treponema antibody and fluorescent amoebic antibody.
5-Eectron Microscopy:
Mainly used for rapid diagnosis of rota virus or herpes infection, CMV in neonatal urine specimens as the virus voids in urine in large amount.

III- Detection of microbial antigens:

Becomes increasingly important in recent years.
- Immunoelectrophoresis . e.g Pneumococcal polysaccharide antigen may be detected in sputum, serum, urine of patients by immunoelectrophoresis, when patients have already given antibiotics where as conventional sputum blood cultures are negative.
- Hepatitis B surface antigen (HbsAg) is commonly detected using ELISA or latex test
-Cryptococcal antigen in CSF in patients with cryptococcal meningitis with latex.
-Rota virus antigen using enzyme linked immunosorbant assay(ELISA) in feces of diarrheic children infected with rota virus.
-Chlamydia trachomatis antigen using ELISA in conjunctival scrappings in patients with active trachoma.
-Detection of *Meningococcae*, *Hemophilus*, *Pneumococcae* antigen in CSF specimens by latex
-Commercially available monoclonal specific antibodies for detection of antigen in clinical specimens or cultures including various Streptococcal, Staphylococcal species, *Neisseria, Candida spp. Chlamydia trachomatis*, and Rotariruses, CMV, Herpes, Adeno viruse, RSV, Influenza viruses.

IV- Isolation of microbes

Is the most reliable way in which a diagnosis can be confirmed and for obtaining antimicrobial susceptibility results.

-Isolation of bacteria or fungus from specimens such as Blood, CSF (which are normally sterile) are easy to interpret. While, bacterial or fungal isolation from specimens collected from sites with normal flora are often difficult to interpret.
-Choice of media is important according to type of specimen and suspected organism.
-Virological or chlamydial isolation methods. Need preparation of the cell line required for support growth of suspected infecting virus.
For Chlamydia -----Vero, Maccoy line
For Adeno - Vero , HEP
For CMV-   Human diploid fibroblast.
For Influenza------ chick embryo.

V-Antimicrobial susceptibility testing

1-Disc diffusion tests

Limitation of disc diffusion tests:-
-Not applied to slowly growing, Fastidious organisms or anaerobes .
-Mycobacterial and fungus susceptibility testing requires specific techniques
-The reported sensitivity tests results not applied to clinical sites infections, e.g *Salmonella typhi* to aminoglycosids.
-Not related to the achieved serum levels or body fluid levels of antibiotics.
-Bacteriostatic measures only.
-Can't be applied to certain antibiotics such as polymyxines.
2-Dilution susceptibility testes:-
Micro minimal inhibitory and minimal bactericidal activity methods.

Methods

-Broth dilution tests
-Agar dilution method.

Application:-

-Serious infection where endpoint concentration is ended
-Disc diffusion yield inter mediate susceptibility
-Life threatening infection due to organisms with unpredictable susceptibility pattern.
-Fastidious or slowly growing organisms.
-Failure of antibiotic therapy
-Serious infections caused by organisms susceptible only to toxic agents

Limitations

-Difficult
It needs the knowledge about the achievable level in serum or body filmed
3- Automated method
4-Antimicrobial concentration gradient methods
-A serial antibiotic dilations are incorporated into the agar.
-E test

VI-Serology

Ten days or longer has to pass before arising antibody demonotrated in chronic infections. e.g , Brucellosis serology is often available
-IgM antibody may indicates recent infection (e.g Rubella)

-ELISA technique widely used nowadays for detection of ab
Immune status of the patients immunization should be taken in considerations.

VII-Molecular biology techniques:

Increasingly important for rapid diagnosis of infections for epidemiological investigations and for monitoring antimicrobial therapy
Also, important for research on the pathogenesis of infection, the developmental new vaccines and immune therapeutic agents.

VIII Gas Liquid chromatographic techniques:

Become increasingly useful for the rapid detection of anaerobic infections
Specimens of pus from abdominal, gynecological or brain abscesses may be shawn to have multiple volatile fatty acids present which indicate anaerobic infection with a few hours of collection of the specimen of pus and this may affect decisions about the chemotherapy of infection.

IX- Skin test:

Of limited value for diagnosis of infection ex:
-Mantaux skin test for tuberculosis.
-Histoplasmin test for Histoplasma infection.
-Casoni test
-Schick test

Classification & pathogenicity of Microbes

The microbial causes of human disease include viruses, Chlamydia, Richettsiae, Mycoplasmas, bacteria, fungi & protozoa. Basic features of these are included in table 1

Table 1. classification Of Microbes

Type of microbes	Nuclic acid	Multiplication		Approx. Size (um)	Seen by LM	Cell wall	Cytmenb.	Senaitive antibiotic	Other features
		Intracellular	Extracellur						
Viruses	**DNA or RNA**	**+**	**-**	**0.01–0.3**	**No**	**No**	**No**	**No**	**Host cell may show inclusion**
Chlamydia esc. Trachomates C. psittaci	**DNA + RNA**	**+Multiplication by binary fission**	**-**	**0.3**	**No**	**No**	**Yes**	**Yes eg tetracycline**	**Host cell may show chonilusin**
Richettsiae eg coxiella, R.prowazeki	**DNA + RNA**	**+ by binary fission**	**-occasional exception**	**0.3**	**Sometimes just visible by sp. Stains**	**Rudimentary cell wall**	**Yes**	**Yes eg tetracycline**	**Typheus transmission by arthropodes.**
Mycoplasma eg. M.pneumonia, M. hominis	**DNA + RNA**	**+ Multiple. efementary badies**	**+**	**0.12–0.3**	**ometines just visible by sp. Stains**	**No**	**Yes**	**Yes eg tetracycline**	**Remarry cells**
Bacteria	**DNA + RNA**	**±**	**+ Multiply binoryfissi**	**0.5 – 0.8 long**	**Yes**	**Yes**	**Yes**	**Yes**	**Rigid cell wall**
Fungi	**DNA + RNA**	**+**	**+**	**Larger than bact. (75 vong, >0.5 wide)**	**Yes**	**Yes (Thicker than bact. Cell + contains sterop)**	**Yes**	**No Sensitive to anti fungal**	**Menles of plant lutno chlorgy**
Protozoa	**DNA + RNA**	**± depends on particular species**	**±**	**Laryer than fungi**	**Yes**	**Yes**	**Yes**	**Not usually**	**-**

Viruses differ greatly from all the other microbes as they consist essentially of only nucleic acid surrounded by a protein coat (capsid) & contain only one instead of two types of nucleic acid.

Chlamydiae & Richettsiae are also obligate intracellular parasites, have both DNA & RNA & multiply by binary fission. Mycoplasmas, bacteria & fungi can be cultured in cell free media unlike the above intra cellular microbes .

Protozoa pathogenic to man are divided into 3 main groups :

1. Sarcodina (amoebae) e g Entamoeba histolytica .
2. Sporozoa eg. Plasmodium falciparum, Toxoplasma gondii.

3. Mostigophora (flagellates) eg Trichomonas vaginalis, Giardia lambilia, Leishmania & Trypansoma sp.

Classification of bacteria :

There are 3 main group of bacteria :

1- Bacteria that are readily Gram – Stained

2- Acid – fast bacilli

3- Spirochetes'

Bacteria that are readily Gram – Stained:

These are classified into Gram positive (blue-purple) or Gram negative (pink-red) cocci or bacilli (Table 2)

After the application of the methyl violet dye, Gram positive bacteria stain blue & this color is retained in spite of decolorization with acetone (or alcohol)and Gram negative bacteria initially stain blue after the methyl violet is applied but the color is lost after the application of acetone (or alcohol). They then take up the pink counter stain (Carbol fuchsine, methyl red or saffronin).

The reason for the difference in color after gram staining is not fully understood, but it is probably related to the large amount of mucopeptide & teichoic acid in the cell walls of Gram positive bacteria. The fact that Gram positive bacteria are more acidic than Gram negative bacteria may account for their greater affinity for a basic dye. Even more important may be the greater permeability of Gram negative cell walls which allow the methyl violet – iodine dye complex to diffuse out after treatment with acetone more readily than cell walls of Gram positive bacteria.

Within each sub group, there are aerobic or anaerobic examples . The majority of bacterial pathogens can grow either aerobically or anaerobically i.e. facultative anaerobes such as *Staphylococcus aureus* or *Escherichia coli* in table 2 these have been included as aerobes. There are a few bacterial species which are strict aerobes such as *Pseudomonas aeruginosa* which will not grow at all anaerobically. Some bacterial species are strict anaerobes such as *Clasteriduim tetani* or Bacteroides fragilis, which will not grow at all aerobically .

Exceptional Gram negative stainable bacteria include *Legionella pneumophillia* & *Borrelia vincent*. *Legionella pneumophillia* requires prolonged staining with the counter stain to be seen in tissues, although it appears readily as Gram negative bacilli in smears made from colonies on agar media. *Borrelia vincent* is the only spirochete pathogen that is easily seen by a Gram stain.

Acid- fast bacilli :

Mycobacteria species are not readily seen by a Gram stain. Zheil-Nelseen (ZN) or other acid- fast stains are required for staining these organisms which have cell walls containing abundant lipids. Examples include *Mycobacterium tuberculosis & Mycobacterium leprae* .

Spirochetes :

They are thin walled spiraled flexible organism which are motile by means of an axial filaments and they are not seen in a Gram stain (except *Borrelia vincent*) but may be seen either by dark ground illumination microscopy or in a silver stain under the light microscope. *Borrelia spirochaetes* in the blood may also be seen in a Giemsa stain.

The three groups of spirochetes include :

1. Treponema : Spirochetes with regular spirals, about 1um apart from each other, 5 – 15um long & about 0.2um wide e.g. *Treponema pallidum* .
2. Leptospira : have tightly spirals, 5 – 15um long & about 0.1um wide. There is often a hooked end eg *Leptospira ictero haemorrhagiae* (Weill's)
3. Borrelia : Large spirochaetes, 10 – 30um long & about 0.3um wide, with irregular spirals 2 – 4um apart from each other eg *Borrelia recurrentis* (a cause of relapsing fever.

Table 2 : Simple classification of Gram stainable bact. pathogens :-

Bacteria	Genus	Sp. examples
G positive Bacteria.		
• ***Cocci*** ○ Aerobic	Clusters …. Staphylococcus	…. *Staphylococcus aureus* *Staphylococcus allbus (S.epidermidis)*
○ Anaerobic	Chains/ pairs …. *Streptococcus pneumoniae* ….. Strept	…. *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus uridans, Enterococcus faecalis* ….. *Streptococcus putridus*
• ***Bacilli*** ○ Aerobic	….. Sporing ….. Non Sporing	…. Bacillus - B.anthracis …..Corynebacterium – C. diphtherum – Listeria – L.monacytogenes – Nocardia – N.asteroides
○ Anaerobic	….. Sporing ….. Non Sporing	…..Clostriduim- C.tetain c.cvelchie (perfrorigens) ….Propionbacterium - P. acnos - Actinomycen - A.israelic
G negative **Bact.**		
• ***Cocci*** ○ Aerobic ○ Anaerobic	… Paris …	… Neisseria – N. meningitis – N. gonorrhoea …Veillonella
• ***Bacilli*** ○ Aerobic ○ Anaerobic	…a. Enterobacteria	e.g. Escherichia – E Coli Klebsiella – K.aerogenes Proteus – P.mirabilis Serratia – S.marcescens Salmonella – S.typhi Shigilla – Sh. sonnei
	b. Pseudomonas	Pseud – P.aeruginosa
	c. Vibrios	Viberio – V. cholera Compylobacter – C. Jejuni
	d. Parvobact	Haemophilus – H.influenzae Brucella – B. abortus Bordetella – B.pertussis Pasterella, yersini –
	e. Legionella	L.pneumophilia –
	f. Spirillum	S.minus
	Amaerabic – Bacteroides –	 B.fragilis

Classification of viruses:

The classification of viruses depends on several factors including the type of nucleic acid present, the arrangement of the capsids into a circular(icosahedral), helical or

complex symmetry, the number of capsomeres, the shape of the virus particle & whether the vision is naked or enveloped .

DNA viruses are:

Pox & papova, Herpes & adeno virus & the remaining viruses & the remaining viruses are RNA viruses .

Classification of fungi :

There are 4 main groups of pathogenic fungi moulels (filamentous fungi) true yeasts yeast – like fungi & dimorphic fungi

1- Filamentous fungi :

Theses grow as long filaments called hyphae & the branched hyphae intervene to form a mycelium. Reproduction is by spores including sexual spores which are used for identification. Culture on Sabaroud's powdery colonies due to the presence of spores eg *Trichophyton mentagrophyets* .

2- True yeasts :

These are unicellular round or oval fungi . Reproduction is by ludding from the parent cell . Cultures creamy colonies e.g. Cryptococcus neoformans .

3- Yeast like fungi:

These are like yeasts since they may appear as round or oval cells & grow by budding . They may also form long non branching filaments known as pseudohyphae e.g. Candida albicans.

4- Dimorphic fungi :

These grow as yeast forms in the body & at 37°C on culture media. They also form mycelia in the environment & on culture media at 22°C eg Histoplasma cupsulatum.

Fungi can also be classified according to whether they cause superficial or deep mycoses in infected patients .

Pathogenesis : Factors affecting the virulence & Spread of Microbes :

The pathogenicity of a microbe depends on host as well as on microbial factors . Host factors include the age of the patient, genetic factors, general host defenses & local host defenses .

Lock's postulates :

1. The particular microbe is always associated with a given disease .
2. The microbe may be isolated in the laboratory from specimens from a patient with the disease .

   It is possible to produce a similar disease in animals by inoculation of the microbe into animals. *Mycobacterium tuberculosis* may be an example where these 3 postulates may be fulfilled.

Factors affecting "Virulence":

The main known factors that affect virulence are concerned with pathogenicity such as toxins & capsules in bacteria . It has also become apparent that the virulence of bacterial stains may also depend on the presence of transmissible genes contained in plasmids or mediated by bacteriophage.

The toxins produced by *Corny diphtheriae* & the erythrogenic toxin produced by *Streptococcus pyogenes* strains in scarlet fever patients are dependent on genes mediated by temperate phages. The fact that particular microbes appear to be more or less virulent at different times, might be due in part to the presence or absence of these types of transmissible genes.

Factors affecting spread:

Epidemiological factors affecting the host are relevant to the spread of microbes including the numbers of susceptible individuals in a geographically defined area, the proximity of the individual to each other & to the source of infection & the presence of other factors necessary for the transmission of infection such as the correct climate or season, the presence of an essential arthropod vector, etc.

Microbial factors that affect the spread depend partly on the virulence of the microbe & partly on the ability of the microbe to survive or multiply in a given inanimate environment or on the hands of patients or hospital staff or in animals arthropods. Carrier states clearly aid the transmission of bacteria. Gram positive bacteria survive well in dry environments while Gram negative & some spirochetes survive lest in moist situations .

Microbes are either transmitted horizontally i.e. between individuals of the same generation (such as the plague bacillus) or vertically i.e. between individuals of different generation (such as cong. Rubella for mother to infant).

Infection either endogenous from the patient's own flora or exogenous from a source outside such as another patient or person, an animal, a vehicle or fomite .

Mode of transmission of microbes include:

1. Direct contact such as N. gonorrhoea .
2. Ingestion such as V. cholerae.
3. Inoculation such as injury transmitting hepatitis B, mosquito bite transmitting malaria .
4. Inhalation such as with measles, Rhino viruses or Mycobacterium tuberculosis.

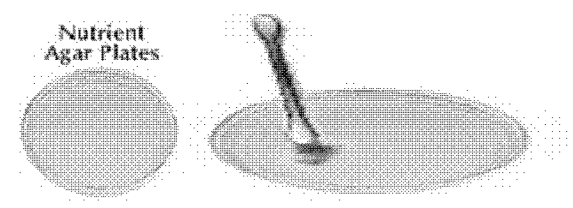
Basic Bacterial Culture and Identification

- Pure Culture Techniques
- Antibiotic Sensitivity Testing
- Gram Staining
- Acid-Fast and Methylene Blue Staining
- Urinary Tract Bacterial Culture

Culture of Normal Flora Organisms

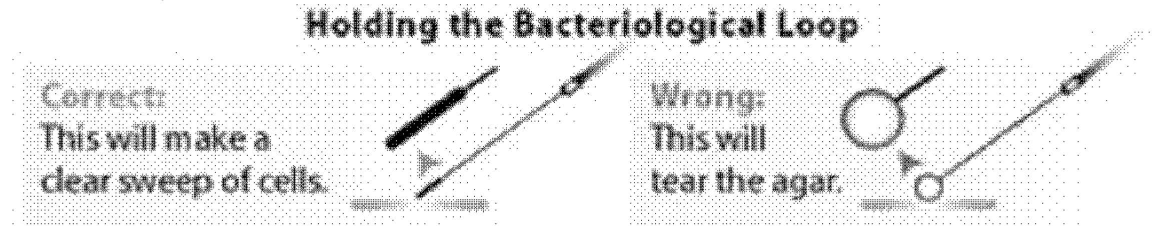
MATERIALS:

- Sterile cotton swab
- Bacteriological loop
- Nutrient Agar plates
- Biohazard discard container

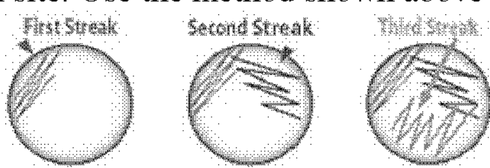


DIRECTIONS:

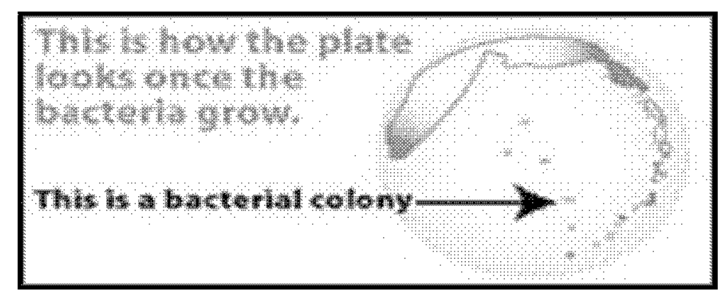
1. Carefully swab the selected skin surface site in an area approximately 4 cm in diameter. Swab thoroughly, rotating the swab to ensure that a good inoculum has been obtained.
2. Transfer the bacteria to the agar culture dish by touching the swab to the agar surface in a single spot. Once you feel confident that a good inoculum has been transferred, the swab can be discarded in the biohazard container.



3. With the inoculating loop, proceed to streak in a second and third streak pattern (see illustration). Briefly, isolate bacterial colonies by pulling several streaks out from the original swab inoculum site. Use the method shown above to avoid tearing the agar.



4. Incubate the plate at 37°C for 24-48 hours.



5. Test the colonies that have grown by Gram staining; identify *Staphylococcus aureus* colonies by testing Gram-positive cocci using a specific latex agglutination reagent that binds to *Staphylococcus aureus*.

Pure Culture Techniques

Microbiologists have developed special techniques and equipment to isolate and grow pure cultures of microorganisms that are free from contaminating forms. This exercise is designed to give the student an introduction to these special techniques. It is important that techniques be practiced until some degree of skill is developed. Pure cultures are essential when identifying unknowns. During the course of the semester, each student will be expected to develop independently a patient-oriented case to correlate with each of 4 separate bacterial isolates. It is important, therefore, to maintain unique isolates in pure culture to be submitted with each case summary. Further instruction will be given during later labs.

THE STREAK PLATE TECHNIQUE

The procedure of streaking a plate with an inoculating loop is used to spread millions of cells over the surface of a solid medium so that some individual cells are deposited at a distance from all others. These cells grow and reproduce, forming an isolated colony. One or more colonies will be well separated from all others and represent a source of a pure culture. The procedure is similar to the one used for streaking from the collection swab.

MATERIALS:

- Streak plates from the previous lab session
- Nutrient agar plates (NA)
- Bunsen burner
- Bacteriological loop

DIRECTIONS:

Examine the streak plates prepared during the previous lab period and locate a number of well-isolated colonies. Now you must transfer a portion of each colony to a separate agar slant. To "pick" a colony you will be using an inoculating loop. Sterilize the loop in the burner flame, let cool 3-5 seconds then touch the end of the loop to the isolated colony, picking up the microorganisms from the colony. Now re-cover the streak plate and pick up one NA plate. You will now be holding the inoculating loop in your right hand and the fresh NA plate in your left (lefties reversed).

Remove the lid from the plate, place the inoculating loop at one edge of the plate and with a sweeping stroke, inoculate the agar using the same tri-streak method as used for the initial isolation. Replace the lid. Flame the loop and proceed to inoculate another

plate from different colonies. Try to use colonies that are visibly different in morphology. Incubate the plates in the 37°C incubator until the next class period.
There is nothing difficult about picking colonies and inoculating slants, but you must avoid contamination.

Identification of Gram-Positive Bacteria

1-Streptococci

DISTINCTIVE CULTURE CHARACTERISTICS

The Streptococci require more nutrients than the Staphylococci. Primary plating of oropharyngeal swab samples should be on BAP (Blood Agar) and CA (Chocolate Agar: heated blood agar). The Nutrient Agar plates (NA) that were adequate for the growth of the Staphylococci are not sufficient to support the group of many of the Streptococci.
β-hemolysis on BAP will be accentuated by stabbing the agar after streaking (increases activity of streptolysin O, which is oxygen labile). Colony morphology will likely be a pinpoint colony appearance. Viridans Streptococci and *S. pneumoniae* will produce α-hemolysis. Gram staining will reveal purple-staining cocci that are smaller than the Staphylococci. *S. pneumoniae* is lancet-shaped and typically occurs in pairs. Streptococci grown in broth culture will appear as long chains of cocci upon Gram-staining. Older cultures may appear Gram-negative due to autolysis.
Most members of this group also require low oxygen tension. This means that they will grow best in the presence of 5% CO_2, a condition that mimics the human body. This can be accomplished either by using a CO_2 incubator or a candle jar placed in a normal incubator. A candle jar is a chamber in which oxygen is depleted by lighting a candle, closing the lid, and allowing the candle to be extinguished when it has consumed the available oxygen. This does not establish an anaerobic condition, merely a microaerophilic one. Stabbing the agar with the innoculating loop after the streak is another technique that facilitates Streptococcal growth.
No special considerations are required for specimen collection and transport when Streptococci are the suspected etiologic agent of infection. Antigen detection methods are most frequently used for identification of patient swab samples, including latex agglutination, and ELISA. The "C" substance recognized by the Lancefield grouping sera is a polysaccharide, and this permits the arrangement of the streptococci into a number of antigenic groups identified as Lancefield group A, B, C, D and so forth. In most clinical laboratories only groups A, B, and D are routinely identified since these groups are responsible for most infections. Culture should be done with negative rapid antigen testing.

APPROACH TO IDENTIFICATION OF THE STREPTOCOCCI

Several methods allow discrimination among the *Streptococcus* spp. The catalase test distinguishes Staphylococci from Streptococci because Streptococci do not produce catalase. Go to Staphylococcal Identification for the catalase test procedure. Once test methods have indicated that the bacteria in question is a *Streptococcus*, a handful of presumptive tests that correlate highly with serological identification methods are typically used to identify bacterial species.

HEMOLYSIS ON BLOOD AGAR

Blood agar is considered to be both a nutritive and differential medium. Many bacteria produce extracellular enzymes that lyse red blood cells present in the agar (hemolysis). The appearance of hemolysis on blood agar plates (BAP) is characteristic for many types of bacteria, especially the pyogenics. Complete clearing in a zone around bacterial colonies is termed βhemolysis. Partial hemolysis produces a zone of greenish tint around the colony (α hemolysis). Bacteria lacking hemolysin produce no halo around the colonies (γ hemolysis or nonhemolysis).

MATERIALS:

- Blood agar plate (BAP) plates
- Sterile cotton swabs in sterile saline

Use the moistened swab to rub gently back and forth with a sweeping motion over the oropharyngeal mucosa. You will probably active the gag reflex, so be quick and careful. Use the swab to deposit the material collected onto the surface of the sterile BAP. Use a flamed and cooled innoculating loop to spread the sample over the plate and accomplish isolating dilution. Incubate for 48 hours at 37°C with a CO_2 environment.

PRESUMPTIVE TESTS FOR GROUP A STREPTOCOCCI

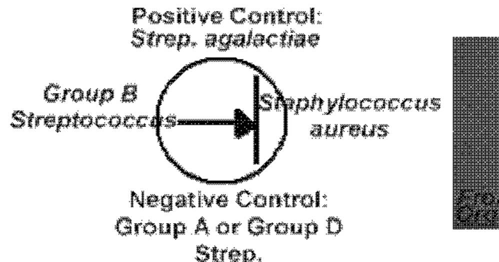
<u>BACITRACIN SUSCEPTIBILITY TEST</u>

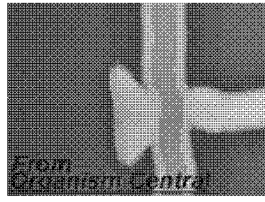
The growth of Group A streptococci is inhibited by a low concentration (0.02 to 0.04 units) of bacitracin in paper disks on blood agar medium, but most other streptococci are not inhibited. Use of low-concentration bacitracin or "Taxo A" disk was previously employed in clinical laboratories for the presumptive Group A streptococci identification. The diameter of a zone of inhibition around the disc indicates sensitivity (>10 mm = sensitive). However, recently it has been recognized that several group C and G *Strep.* are also sensitive to bacitracin.

The PYR test uses L-pyrrolidonyl-α-naphthylamide substrate to detect the presence of the enzyme L-pyrroglutamyl-aminopeptidase. This test is considered to be more reliable than bacitracin sensitivity. It is performed in much the same way as <u>the oxidase test</u>, using a colony smeared on filter paper impregnated with the substrate. A drop of indicator reagent should produce a red color within 5 min. if the colony is *Streptococcus pyogenes*.

PRESUMPTIVE TESTS FOR GROUP B STREPTOCOCCI

<u>The CAMP Test</u>





The laboratory identification of Group B hemolytic streptococci has been simplified by implementation of the CAMP test which is easy to perform and well within the capabilities of small laboratories.

PRINCIPLE:

The hemolytic activity of staphylococcal-hemolysin on red blood cells (RBC) is enhanced by an extracellular factor produced by Group B streptococci, called the CAMP factor. Therefore, wherever the two reactants overlap, an accentuation of the ß-hemolytic reaction will be noted.

PROCEDURE:

The CAMP test is performed by making a single streak of the streptococcus perpendicular to a strain of *Staphylococcus aureus* that is known to produce ß-hemolysin. The two streak lines must not touch one another. The inoculated plate must be incubated with an ambient atmosphere (room temperature). The plate **should not be incubated in an anaerobic environment** because many Group A streptococci are positive in the absence of O_2. Any Bacitracin-negative, CAMP-positive, bile-esculin-negative streptococcus can be reported as: Group B streptococcus, presumptive by CAMP.

Positive control: *S. agalactiae*.

PRESUMPTIVE TESTS FOR GROUP D STREPTOCOCCI

Lancefield Group D streptococci are divided into two groups: (1) Enterococci, and (2) nonenterococci. *Enterococcus faecalis* and *Enterococcus faecium* are the species of *Enterococcus* most commonly isolated in laboratories which identify Enterococci. However, many laboratories do not attempt to differentiate them into species, but simply report "*Enterococcus*". Similarly, *Strep. bovis* and *Strep. equinus* may not be differentiated, but reported as "Group-D streptococci, not Enterococci".

Bile-Esculin Hydrolysis Test

The purpose of this test is to determine the ability of an organism to hydrolyze the glycoside esculin to esculatin and glucose in the presence of bile (10 - 40%). This test aids in the differentiation of group D streptococci from other "not group D streptococci".

PROCEDURE:

1. Inoculate the organism to be tested into the bile esculin medium. Incubate at 37°C for 24 hours (stab into medium, then streak on slant).

INTERPRETATION:

Positive Test: Presence of a black to dark brown color on the slant (half or more of the medium is blackened) -- (*Enterococcus faecalis*)

Negative Test: No blackening of the medium OR blackening of less than half the tube after 72 hours of incubation -- (*Streptococcus agalactiae*)

Salt Tolerance Test for Enterococci

This test is based on the ability of the enterococci to grow in 6.5% NaCl and separates them from the "Group-D streptococci, not enterococci".

PROCEDURE:

Inoculate a loopful of organism to be tested into the 6.5% NaCl broth and incubate at 37°C for 18 hours (overnight).

Positive control: *Enterococcus faecalis*

2-Normal Flora Staphylococci

FERMENTATION ON MANNITOL SALT AGAR (MSA)

MSA is a selective medium for the recovery of staphylococci from mixed cultures. This medium takes advantage of the ability of staphylococci to grow in the presence of 7.5% NaCl and differential fermentation of mannitol. A pH indicator (phenol red) is incorporated into the agar. The agar color turns from deep pink to yellow in the presence of acidic fermentation products. The test is diagnostic for *S. aureus*: growth positive + fermentation positive = *Staphylococcus aureus*.

MATERIALS:

- MSA plates
- Pure cultures from the previous lab period, one confirmed *Staphylococcus aureus* by latex agglutination; one *Staphylococcus* culture confirmed NOT to be *S. aureus* by latex agglutination. Both should be confirmed Gram-positive by staining.

Streak the two *Staphylococcus* cultures on opposite sides of an MSA plate. Incubate at 37°C and observe agar appearance after 24 hours. DO NOT INCUBATE FERMENTATION TESTS IN THE PRESENCE OF CO_2, BECAUSE THE COLOR CHANGE WON'T WORK.

CATALASE TEST

Discriminates between *Staph* and *Streptococcus* species. The basis for the test is:

H_20_2 + catalase = H_20 + 0_2 (bubbled off)

MATERIALS:

- Pure cultures from the previous lab period, one confirmed *Staphylococcus aureus* by latex agglutination; one *Staphylococcus* culture confirmed NOT to be *S. aureus* by latex agglutination. Both should be confirmed Gram-positive by staining.
- Glass microscope slides
- 1 tube containing 3% H_20_2

PROCEDURE:

1. With an inoculating needle, pick the center of an 18 to 24 hr pure colony and place on a clean glass slide.
2. Test is not reliable if blood agar is introduced into the H_20_2.
3. Add a drop of 3% H_20_2 to cover the organism or slide.
4. Observe for immediate bubbling (gas liberation); record the results.

COAGULASE TEST

The laboratory test based on detecting the production of the enzyme coagulase by *Staphylococcus* species is used to differentiate between staphylococci and streptococci, as well as between coagulase-negative staphylococci and *Staphylococcus aureus*. Coagulase is secreted extracellularly by the bacteria and reacts with the coagulase-reacting factor present in plasma. Clot formation in the plasma is mediated by the complex formed in this reaction.

MATERIALS:

- 2 tubes of citrate plasma
- Microscope Slides
- Pure cultures from the previous lab period, one confirmed *Staphylococcus aureus* by latex agglutination; one *Staphylococcus* culture confirmed NOT to be *S. aureus* by latex agglutination. Both should be confirmed Gram-positive by staining.

1. Pick a colony of putative staphylococci using the inoculating needle. Emulsify the colony in citrate plasma. Repeat for a second organism (one not suspected to be a staphylococcus).
2. Cover the tube and incubate for **4 hours** at 37°C. Observe for clot formation by gently tipping the tube.
3. If no clot has formed, incubate the tube again for **18 hours** and observe. Weak coagulase producers may require this prolonged incubation period to develop clots.

HEMOLYSIS ON BLOOD AGAR (BAP)

MATERIALS:

- BAP plates
- Pure cultures as above, one confirmed *Staphylococcus aureus* by latex agglutination; one *Staphylococcus* culture confirmed NOT to be *S. aureus* by latex agglutination. Both should be confirmed Gram-positive by staining.

Streak the two organisms on opposite sides of the BAP plate. Incubate the plates for 24-48 hours at 37°C in the presence of a 5% CO_2 atmosphere. Observe the zone of color change around the colonies.

Culture and Identification of Fastidious Bacteria

Neisseriae and *Haemophilus* Species

***Neisseriae* & GONORRHEAL DISEASES**

Diseases: Gonorrhea.

Etiologic Agents: *Neisseria gonorrhoeae*

Source: Human genital tract by sexual contact or by birth.

Pathogenesis: Infections of mucous membranes lined by nonsquamous epithelium. Local epithelial cell destruction with PMNs.

Laboratory Diagnosis: Isolation of etiologic agent. Gram stain. Antigen detection by enzyme immunoassay. Several manufactures have identification kits that give rapid results like Dupont's "*Gonocheck II*"

NEISSERIAL DISEASES (other than gonorrhea)

Diseases: Megingitis (epidemic CNS fever), acute otitis media, maxillary sinusitis, bronchopulmonary infections

Etiologic Agents: *Neisseria meningitidis, lactamica & sicca; Moraxella catarrhalis*

Source: Commensal bacterial flora of the oropharynx, human respiratory aerosol transmission, acquired by inhalation.

Pathogenesis: *Neisseria meningitidis* - Central nervous system responses reflect acute inflammatory changes. Septic manifestations include disseminated intravasuclar coagulation and damage leading to shock.

Laboratory Diagnosis: *M. catarrhalis* infections - Gram-stained smear and culture; *Neisseria* spp. - Gram stain and culture from blood, CSF, or transtracheal aspirates isolation.

Adapted from Laboratory Diagnosis of Infectious Diseases, ed. A. Balows

Representative of the genus *Neisseria* are Gram-negative cocci which are **oxidase positive and catalase positive**.

Materials:

Neisseria lactamica culture BAP and Chocolate agar plates

Oxidase & Catalase reagents Nasal culture (sterile swab needed)

I. Morphological studies:

STAINING CHARACTERISTICS:

1. Prepare a Gram stain of these organisms.
2. Study the morphological and staining characteristics.

CULTURE:

1. Streak the nasal swab for the isolation of Neisseria spp. from the normal flora on each of BAP and Chocolate agar plates.
2. Incubate in CO_2 incubator at 37°C for **24 hours**.
3. Study and record the colony characteristics.

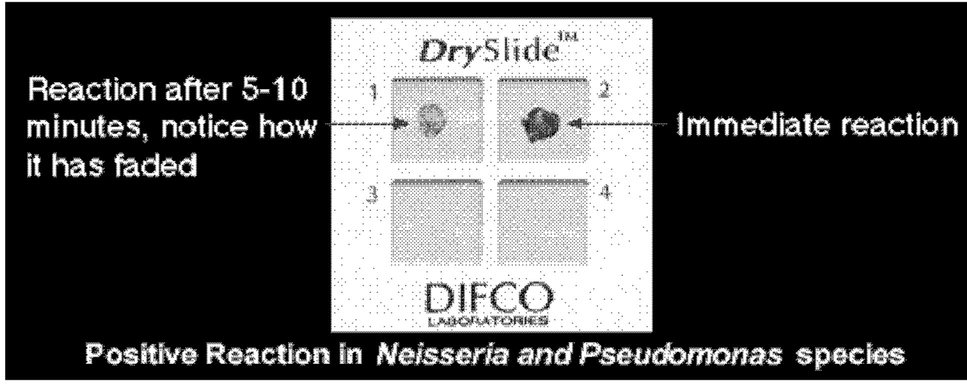
II. Identification:

A. Oxidase Test

This laboratory test is based on detecting the production of the enzyme cytochrome oxidase by Gram-negative bacteria. It is a hallmark test for the *Neiserria*. It is also used to discriminate between aerobic Gram-negative organisms like *Pseudomonas aeruginosa* and other *Enterobacteriaciae*.

MATERIALS:

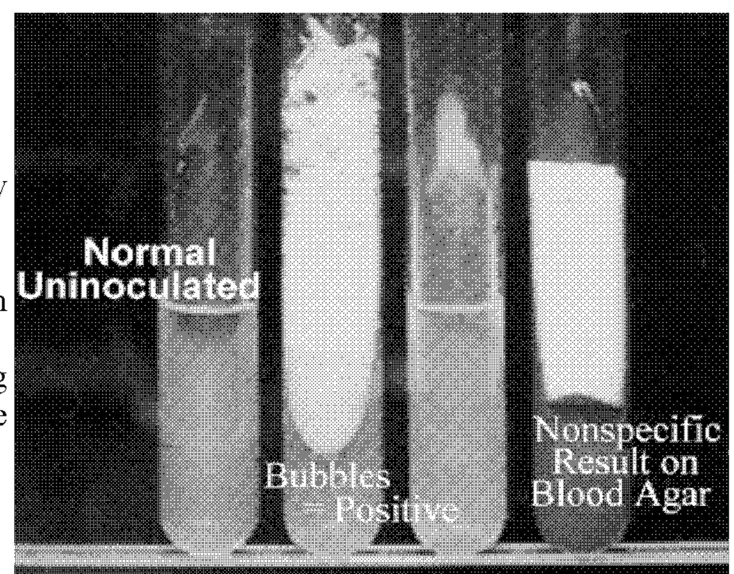
- Bacterial colonies from nasal or oropharyngeal swab culture growing on Chocolate Agar Plates (CA)
- Oxidase reagents
- Sterile toothpicks
- *Neisseria* culture and an unknown culture



PROCEDURES:

1. Pick a colony for testing using a sterile wooden toothpick. Transfer the colony to the surface of one of the four grid areas on the Oxidase Test slide.

B. Catalase test
1. Transfer a large isolated colony from the culture dish to a clean microscope slide.
2. Add two drops of 3% hydrogen peroxide reagent to the colony.
3. Observe for immediate bubbling (gas liberation) and record the result.



2. The test slide surface is impregnated with the reagent tetramethyl-*p*-phenylenediamine dihydrochloride. This reagent causes a dark purple color to appear in the presence of cytochrome oxidase.
3. Observe the color change. The reaction color will change from pink to maroon to dark purple.
4. Read the test results within 10 seconds. Some organisms may show slight positive reactions after this period and such results are NOT considered definitive.

Haemophilus influenza DISEASES

Diseases: Pneumonia, meningitis, epiglottitis, septic arthritis, cellulitis, otitis media, conjunctivitis, neonatal infection, and other clinical syndromes

Etiologic Agents: *Haemophilus influenza* (both serotypable and nonserotypable).

Source: Human respiratory and genital tract by direct contact or inhalation.

Pathogenesis: Infection by serotypable *H. influenza* results in a primary bacteremia followed by acute pyogenic infection. Infection by nonserotypable *H. influenza* results in isolated mucosal infection with an occasional secondary bacteremia.

Laboratory Diagnosis: Laboratory cultures of organisms from blood, CSF, joint fluid, and biopsy material.

INFECTIONS BY *Haemophilus* SPECIES OTHER THAN *H. influenza*

Diseases: Conjunctivitis, genital ulcer, endocarditis, brain abscess, pneumonia, and other syndromes.

Etiologic Agents: *H. parainfluenza* and other *Haemophilus* species.

Source: Human respiratory and genital tract by direct contact or inhalation.

Pathogenesis: Acute pyogenic infections

Laboratory Diagnosis: Isolation of the organism from blood, sputum, tissue or mucosal swab.

The bacterial species in this group can cause serious respiratory infections. *Haemophilus* also has species commonly found in the **normal flora**.

The genus *Haemophilus* contains a number of species of **fastidious, Gram-negative coccobacilli**. Gram-stained preparations of infected clinical materials show pleomorphic threadlike filaments, commonly admixed with the coccobacillary forms. These fastidious organisms require specially enriched culture media such as chocolate agar and microaerophilic conditions of incubation. Culture on both BAP and chocolate agar (heated blood agar), with growth only on the chocolate agar is characteristic of the *Haemophilus* group organisms. The use of blood agar also allows differentiation of *H. haemolyticus* from *H. influenzae* based on hemolysis produced by the former. Some species require Factor X, a heat-stable derivative of hemoglobin; others require NAD, also known as Factor V. Factor V is heat-labile. It can be derived from extracts of yeasts and is produced by certain bacteria such as *Staphylococcus aureus*. *H. influenza* requires both X and V factors. These culture characteristics will be reinforced during the satellitism demonstration (see next page).

CULTURE OF *Haemophilus* SPECIES

MATERIALS REQUIRED:

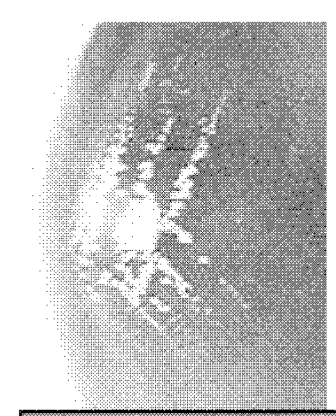
Haemophilus influenzae 2 BAP plates

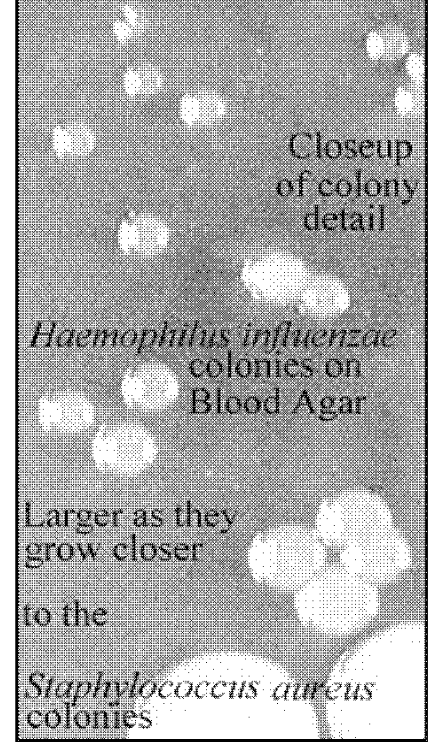
Haemophilus parainfluenzae 2 Chocolate agar plates

Streak blood agar and chocolate agar plates with each species. Incubate within a candle jar at 37°C overnight or longer. Note differences in the two media's ability to support growth, and in colony morphology.

Staphylococcus Streak Technique (Satellitism)

V and X Factor Production: Strain Identification





Material required:
Haemophilus influenzae
Haemophilus parainfluenzae
Staphylococcus aureus
1 BAP

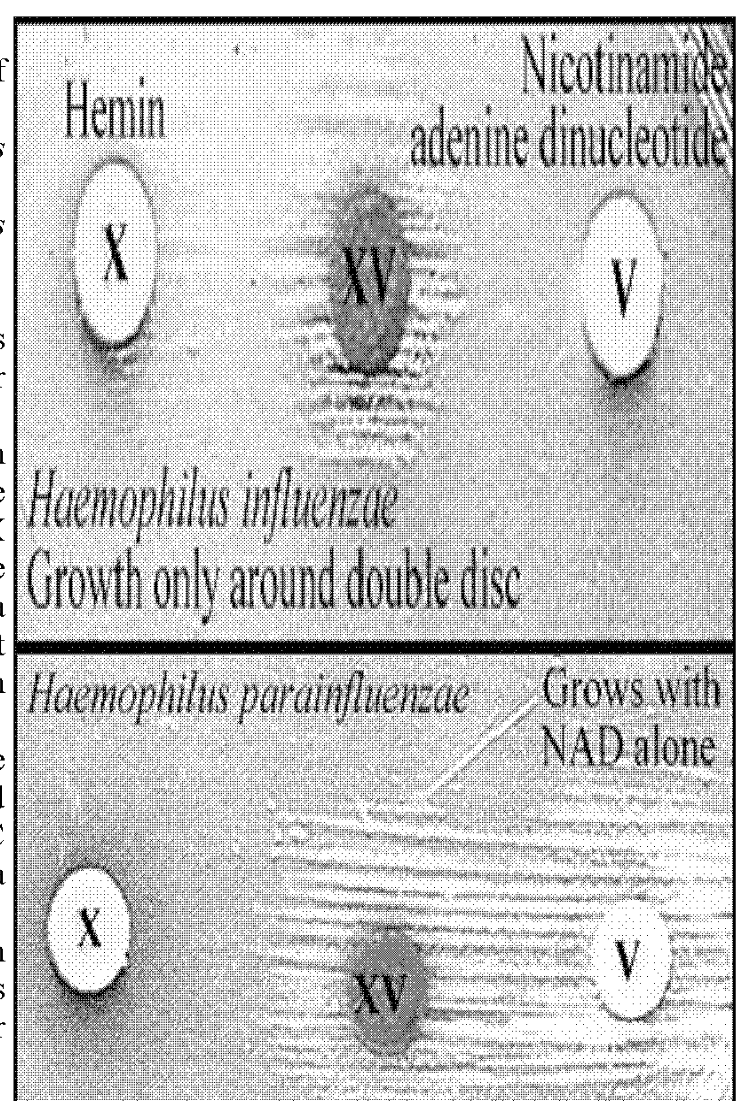
PROCEDURE:

1. *H. influenzae* should be heavily streaked on a BAP.
2. Using an inoculating wire, a single narrow streak of a hemolytic S*taphylococcus* known to produce NAD (factor V) should be made through the area where the *H. influenza* had been inoculated.
3. Incubate plates in a candle jar at 37°C for **24 hours**. Observe the results and record below:

Material required: (each group of four students)
2 NA plates *Haemophilus influenzae*
X, V & XV discs *Haemophilus parainfluenzae*

PROCEDURE:

1. Each of the *Haemophilus* strains should be inoculated heavily for confluent growth on the NA plates.
2. Divide each streaked plate in thirds (draw sections on the plate bottom with a marker). Place an X disc at the center of one of the thirds, a V disc at the center of a second section, and an XV disc at the center of the third section (place discs on the agar surface).
3. Repeat the procedure for the second strain. Both plates should be incubated in a candle jar at 35°C for **24 hours** (this produces a microaerophilic condition).
4. A characteristic growth pattern should be the result of this exercise. Record your observations.



Identification of the *Enterobacteriacae*

THE ENTERIC BACILLI

Diseases: Enteric bacteriosis (hospital-acquired or nosocomial infections with various Gram-negative rods acquired by patients during hospitalization).

Etiologic Agents: *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, and *Serratia*.

Source: environmental sources, clinical sources, and normal flora

Pathogenesis: Bacteremia, pneumonia, urinary tract infections, wound infections, CNS infections, abscess formation in various organs, and colonization and infection of implants, prostheses, and catheters.

Laboratory Diagnosis: Bacteriologic isolation of etiologic agents from blood, other body fluids, wounds, and exudates. Antimicrobial susceptibilities tests characteristically reveal resistance to commonly used antibiotics. Molecular epidemiological analysis may identify resistance plasmids. Several companies manufacture diagnostic kits based

on biochemical test properties of the organisms. The Enterotube method will be demonstrated.

THE LACTOSE FERMENTERS

***E. coli* INFECTIONS**

Diseases: Intestinal infections (diarrhea, dysentery, hemorrhagic colitis). Urinary tract infections (asymptomatic bacteriuria, cystitis, pyelonephritis, prostatitis). Bacteremia. Neonatal meningitis.

Etiologic Agents: Intestinal infections - enterotoxigenic *Escherichia coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterohemorrhagic *E. coli* (EHEC). Urinary tract infections - *E. coli*, usually strains carrying uropathogenic determinants. Bacteremia - *E. coli*, usually strains belonging to the same serogroups associated with urinary tract infections. Neonatal meningitis - *E. coli (*usually strains with K1 Ag).

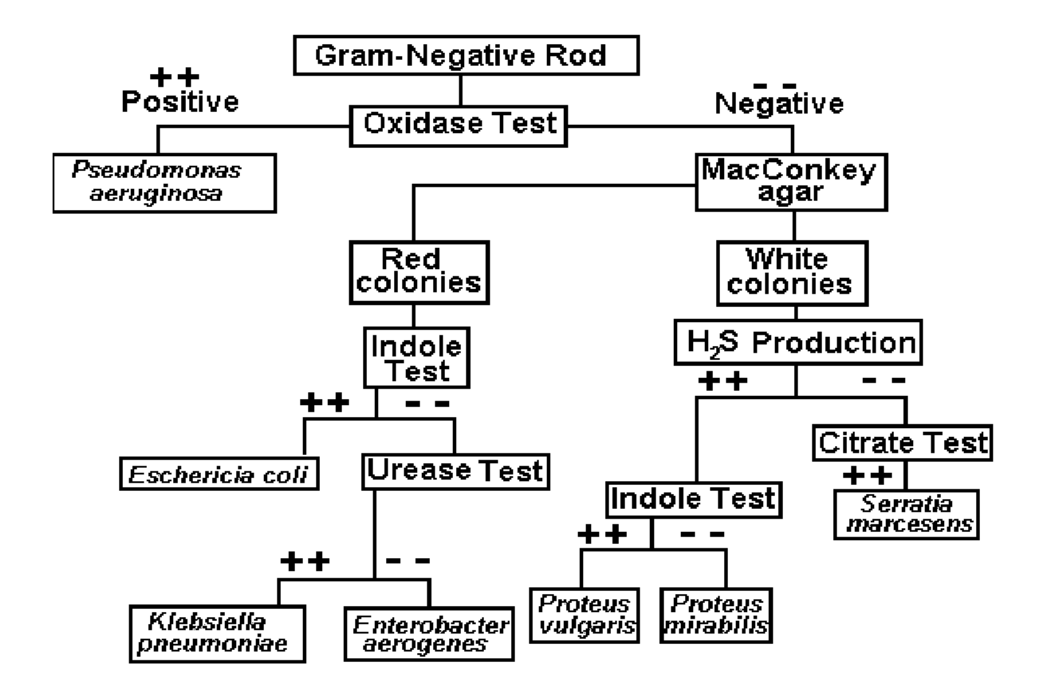
Source: Intestinal infections - human intestinal tract, cattle (EHEC). UTI, Bacteremia and Neonatal meningitis- human intestinal tract

Pathogenesis: Intestinal infections - interaction of the intestinal mucosa with colonization factors, heat-labile (LT) and heat-stable (ST) enterotoxins (ETEC); enterocyte invasiveness factors (EIEC); enteroadhesiveness factors. Urinary tract infections - interaction of urinary tract mucosa with adhesins (type 1 fimbriae, P-fimbriae, X-adhesins); possibly hemolysin, O polysaccharides; immature host immunity; shock associated with endotoxin. Neonatal meningitis-acidic polysaccharide capsule K1.

Laboratory Diagnosis: Isolation of *E. coli* from the appropriate clinical samples. Kits are available commercially. These are based on the biochemical tests that will be presented in this lab section.

Adapted from Laboratory Diagnosis of Infectious Diseases, ed. A. Balows

A SIMPLIFIED BRANCHING FLOW DIAGRAM FOR GRAM-NEGATIVE RODS



ISOLATION OF *ENTEROBACTERIACEAE*

There are numerous plating media in use today, some selective, and others inhibitory. Most laboratories prefer to employ one selective medium such as SS agar, and one inhibitory medium, such as EMB or MacConkey agar for fecal specimens. For specimens other than feces or rectal swabs, a combination of MacConkey or EMB agar together with a BAP plate is usually sufficient.

Characteristics and Tests Used for the Species Identification of Enterics:

Identification of the *Enterobacteriaceae* is based on colony morphology on primary isolation media and on biochemical reactions. The following set of tests is a subset of those tests widely used in clinical laboratories:

- Utilization of **carbohydrates** (**fermentation**)
- The **IMVC** reactions:

I = production of **Indole** from tryptophan

M = **Methyl Red** Reaction

V = **Voges-Proskauer** test (Production of acetyl-methyl carbinol)

C = Utilization of **Citrate**

- **Motility**
- **Production of Urease**
- **Production of H_2S gas**

Carbohydrate fermentation pattern for an isolate can be determined using any of several commercially-available minaturized test configurations (Enterotube, API test strip). In

the clinical lab these tests are usually performed using a Vitek automated analyzer and liquid culture isolates.

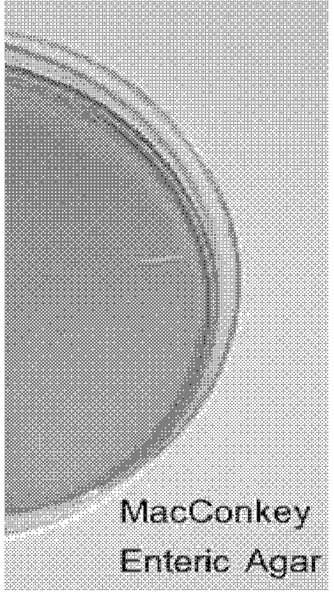
Automated Analysis System for Enteric Microorganisms

Hospital microbiology laboratories typically employ fully automated computerized methods for the identification and classification of enteric bacterial species. One example of such a system is the Automicrobic System (bioMerieux Vitek, Inc. Microscan) The system uses disposable polystyrene cards that include 30 reaction micro-wells. Each microwell contains biochemical substrates or dehydrated antibiotics. A filling module automatically fills each microwell with the test bacterium and seals the card. An incubation and reading module optically scans the cards on a 1-hour rotational basis as they are incubated. A computer module records the biochemical reactions and performs identification analyses. The final identification report can be available with as little as 6 hours of incubation. The total time required to culture and identify a suspect enteric pathogen, however, includes the initial primary culture for isolation, generally on MacConkey agar.

I) **Primary isolation medium** **(Inhibitory)**.

Important Purpose and Differential Medium Ingredients

MacConkey agar



Uninoculated MAC Plate

MAC Agar Formula

Bile salt (1.5g/l) & Crystal violet

Purpose: Recover the *Enterobacteriaceae* & related Lactose-fermenting Gram-negative enteric bacilli.

Inhibitor: Bile salt & Crystal Violet. Inhibit the growth of Gram-positive bacteria and some fastidious Gram-negative bacteria.

Carbohydrate Source: Lactose
Neutral Red, pH indicator

Eosin-Methylene-Blue agar (EMB)

Eosin Y and Methylene-blue
Purpose: Isolation & detection of the *Enterobacteriaceae* or Lactose-related coliform bacilli from specimens with mixed bacteria.
Inhibitor: Eosin & methylene blue. Inhibits the growth of Gram-positive bacteria and some fastidious G- organisms
Carbohydrate Source: Lactose
pH Indicator: Eosin & methylene blue combine to form a precipitate at acidic pH.

Differential and Selective Culture of Bacteria

The Selective Media allow one type of organism to grow preferentially because of the components included in the agar. Examples are the agars that selectively inhibit the growth of most organisms, while allowing skin bacteria to grow (Mannitol-Salt Agar for Staph. culture), those that inhibit Gram-positive organisms due to bile salts (MacConkey agar) and dyes like basic fuchsin, eosin, and methylene blue (EMB agar).
The Differential Media allow different groups of bacteria to be distinguished based on their appearance and/or biochemical reactions. Blood agar (BAP), as described for the culture of Streptococci, is one such agar. It allows bacteria to be distinguished based on hemolysis of the blood in the agar. However, it does not prohibit the growth of other types of bacteria, so it is not a selective media. Some strains of Staphylococcus produce beta-hemolysis on BAP.
Some differential media are sophisticated enough to allow preliminary identification of bacterial species to be made simply from culture results. MacConkey agar is both differential and selective. Since it contains lactose and neutral red dye, so lactose-fermenting colonies appear pink-to-red in color and are easy to distinguish from nonfermenting colonies. This is because of a reaction between the acid metabolic by-products of bacterial growth and the agar components.

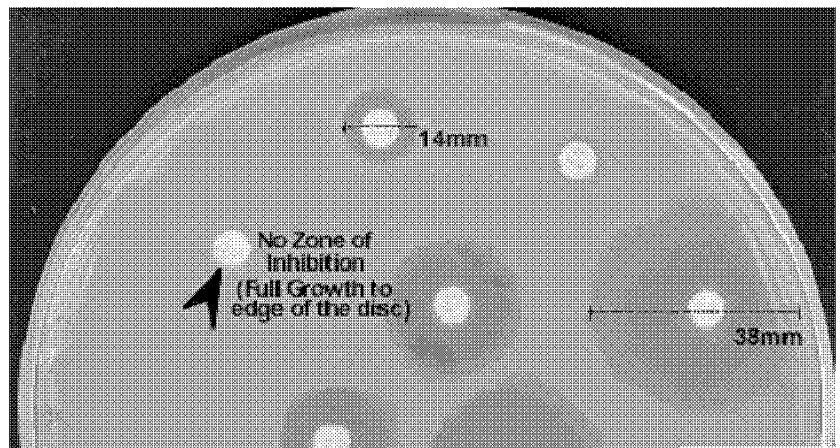
TESTS FOR SUSCEPTIBILITY TO ANTIMICROBIAL AGENTS

Bacteria generally can be grouped with respect to antibiotic susceptibility. Gram positive bacteria are more permeable to many of the most common antibiotics, probably because they lack the outer membrane of the Gram negatives. This makes them more susceptible as a group to the actions of antimicrobial agents. However, generalizations concerning susceptibility and resistance are difficult to make because of the vast differences in bacterial species. In addition, the emergence of new antibiotic resistant strains in particular environments makes it increasingly important to adequately monitor resistant strains. On a global scale, outbreaks of Shigella, E. coli and Salmonella carrying drug-resistance plasmids have emphasized the need to monitor drug resistance within populations and to make this information widely available.

KIRBY-BAUER DISC METHOD

One simple and commonly used assay for microbial susceptibility is the disc diffusion or Kirby-Bauer method, which will be demonstrated. Antibiotic-impregnated discs release antibiotic into the surrounding medium when placed on the surface of solid agar.

Untreated agar plates are inoculated with test microorganisms and antibiotic discs are placed on the agar surface. Disc diffusion is a qualitative method based on an approximation of the effect of antibiotic on bacterial growth on a solid medium. A zone of growth inhibition around the antibiotic disc will occur if the organism is susceptible to the antibiotic.



As the distance from the disc increases, there is a logarithmic decrease in antibiotic concentration. Standardized regression curves have been developed that correlate inhibition zone size to the minimum inhibitory concentration of the antibiotic. However, even though it is the most common test for antimicrobial effectiveness, not all bacteria can be tested using disc diffusion.

MATERIALS:

- Bacterial cultures
- Sterile loops
- Antibiotic discs
- Agar plates
- Bunsen burner
- Striker

STUDENT DIRECTIONS:

1. Light the bunsen burner and let it burn for five minutes to create a clean work environment.
2. With an inoculating loop, pick one colony from the plate you inoculated during the previous class. You will use it to grow a lawn of tiny bacterial colonies.
3. Use the loop to distribute the bacteria completely over the surface of the agar. Where you previously needed to streak for isolated colonies, now you are streaking for maximum distribution over the surface of the agar. Turn the plate 90^{o} and streak the entire surface again.
4. Repeat step #3 until you are satisfied with the amount of bacteria you have deposited on the agar.
5. Use the antibiotic disc dispenser to release several discs onto the agar surface. Press down with another bacteriological loop to secure.

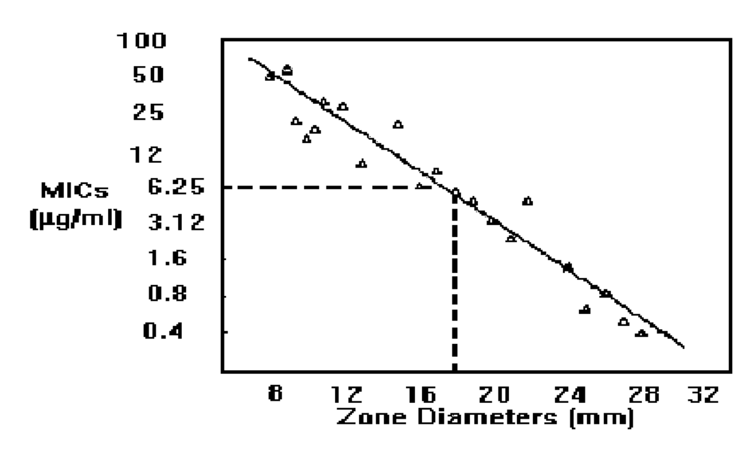
6. Observe the growth pattern after at least 24 - 48 hours of growth (standard read time is 48 hours).

MINIMUM INHIBITORY CONCENTRATION (MIC) and MINIMUM BACTERICIDAL CONCENTRATION

The minimum inhibitory concentration (MIC) method uses doubling dilutions of antibiotics solubilized in bacteriological medium to assess the susceptibility of specific bacteria to the antimicrobial agents selected. The MIC test measures the lowest concentration of an antibiotic that inhibits the visible growth of test microorganisms. In practice, microtiter plates containing prediluted antibiotics in lyophilized form are reconstituted and incubated along with test bacteria.

Once an MIC has been performed a minimum bactericidal concentration (MBC) can subsequently be determined. The MBC is set up with subcultures made from each MIC well that appears visually clear. The MBC is the concentration of antibiotic included in the first tube from which colonies calculated as less than 99.9% growth is recovered.

Comparison of Kirby-Bauer and MIC test results:



Bacterial Staining

GRAM STAIN

Gram stain exploits the basic differences in the outer layers of bacteria so that certain bacterial groups retain the initial stain while others readily lose this dye during the decolorization process. Bacteria that decolorize will be stained the color of the counterstain. The first two clues to the identification of any bacterium is the shape of the organism and the Gram staining characteristic. In fact, in some situations need no further information is needed (at least at the beginning) and you will come across microbiologists referring toan isolated organism as a "Gram-negative rod" or a "Gram-positive coccus". With more experience in microbiology, this information actually tells you a great deal about the genus to which the organism belongs.

MATERIALS:

- Gram stain solutions
- Microscope slides
- Agar plate cultures of *Escherichia coli* and *Staphylococcus aureus*

DIRECTIONS:

To perform the Gram stain, a good smear is absolutely essential. Too thick a smear will produce a slide giving false positive reactions. Too thin a smear will require lots of time seeking organisms on the slide.

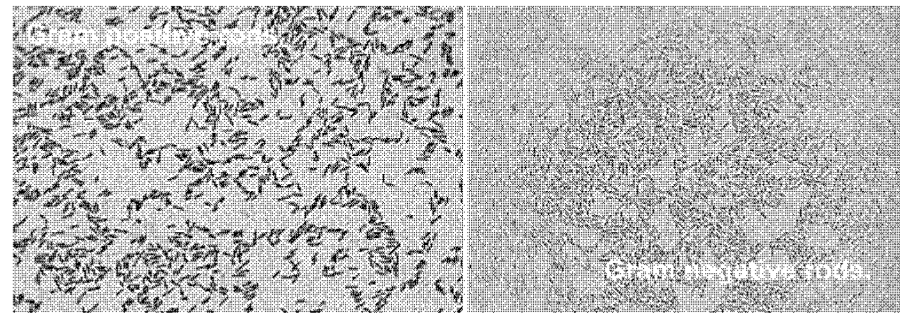
1. Prepare a good smear

- Place one drop of water on a microscope slide.
- With an inoculating loop, pick one colony; place colony in the drop of water and make a milky suspension.
- Allow the smear to air dry.
- Heat-fix the smear by passing the slide over the burner flame several times. Take care not to overheat (overheating will lyse the cells). Test the temperature by placing the slide on the back of your hand. If it's too hot for your hand, it's too hot for the bacteria.

2. Flood the slide with crystal violet stain and leave for 1 minute.
3. Pour off the crystal violet stain, wash with water, and flood the slide with Gram's iodine solution. Allow a contact time of 1 min.
4. Gently rinse the slide with water from a squirt bottle.
5. Blot the slide dry to remove excess water with a Kimwipe.
6. Holding the slide on a slant, flood the slide with the combination 95% acetone-alcohol/safrinin solution, watching for the dye to continue washing from the cells. As soon as most of the crystal violet dye stops leaching from the cells, gently wash the slide in water to stop the decolorization process. Be careful not to overdo this step.
7. Blot the slide dry or allow to air dry.

The critical step is #6, the decolorization process. In all probability it will take some practice to train your eye to detect when the dye has stopped leaching from the cells. Too thick a smear will protect cells lying beneath the top cells from becoming decolorized. In this case a Gram negative organism will appear Gram positive. Many Gram-positive cultures will appear Gram-negative or Gram-variable if the cells are over-decolorized. Is it very likely that Gram-negative cocci would be cultured from a normal skin swab?

You should start this exercise by preparing a slide with 2 smears, one from a Gram positive coccus (off your normal flora plates) and the Gram negative rod (*Escherichia coli*). The presence of the two smears adjacent to one another will not affect the Gram stain, but it will let you see the difference in decolorization between the two organisms. After staining one slide, examine the results under the microscope using the oil immersion objective. If you fail to see the red-pink rod-shaped *Escherichia* or if the cocci are not a deep purple, try the stain again with another slide--this time correcting the decolorization step (longer or shorter). A *Candida albicans* culture is also interesting to examine in comparison with Gram-positive bacteria.



Other Bacterial Staining Methods

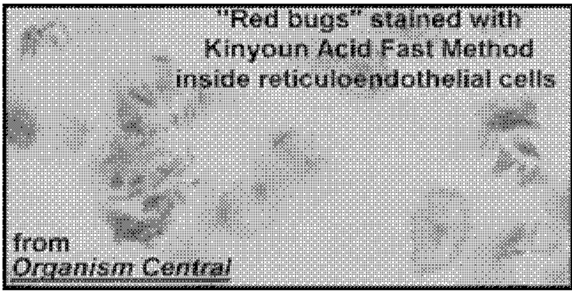
THE ACID-FAST STAIN

The property of acid-fastness, which is detected by the acid- fast stain, is primarily of clinical application to detect members of the genus *Mycobacterium. M. tuberculosis*, the etiologic agent of tuberculosis, is the most common pathogen of this group. Other microorganisms, particularly the *Nocardia*, can be identified by their acid-fast characteristic. The term acid-fast is derived from the resistance displayed by acid-fast bacteria to decolorization by acid once they have been stained by another dye.

Cultures provided:

MATERIALS:

- *Mycobacterium phlei* culture
- Prepared Kinyoun carbol fuchsin stain
- 3.0% acid-alcohol (70% ethanol containing 0.5% hydrochloric acid)
- Methylene blue counterstain



DIRECTIONS:

1. Prepare a bacterial smear in the conventional manner (as for Gram-staining), air-dried and heat fixed. Flood the smear with Kinyoun carbol fuchsin reagent and allow to remain in contact for at least 5 min. Do not allow the stain to dry out - add more if needed.
2. Rinse the excess stain off with deionized water from the squirt bottle. Tilt the slide to drain water.
3. Decolorize the smear with acid alcohol until the color no longer runs from the smear. It is difficult to overdecolorize acid-fast bacteria.
4. Wash the smear with deionized water (use the squirt bottle).
5. Counterstain for 30 sec -- 1 min. with 1% methylene blue.

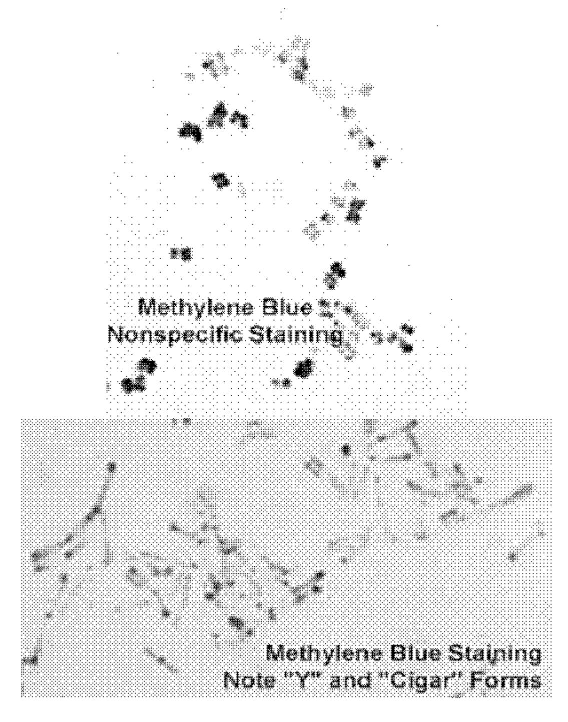
6. Rinse thoroughly, blot dry, and examine under oil immersion.
7. Study the stained preparation using the light microscope. A blue-stained mass can usually be found on low-power. Examine the mass with the oil-immersion lens. On high power, acid-fast bacteria will appear an intense, crimson red (retaining the carbol fuchsin), while the remaining material will be blue from the counterstain.

THE METHYLENE BLUE STAIN

The Methylene Blue Stain is considered to be a simple stain, in contrast with the Gram and Acid-Fast Stains, which require a counterstaining step. Methylene Blue Stain is especially useful for observing metachromasia in bacterial species such as *Corynebacteria*.

MATERIALS:

- 1% Methylene blue stain solution
- Glass microscope slides
- normal flora cultures



DIRECTIONS:

Prepare a bacterial smear, as above.

1. Flood with methylene blue solution.
2. Leave stain in contact with the smear for 30 sec -- 1 min.
3. Rinse thoroughly, blot dry, and examine under oil immersion.

Sampling for FUNGAL Infections and Culture

Skin Scrapings and Swabs

In patients with suspected tinea or ringworm any ointments or other local applications present should first be removed with an alcohol wipe. Using a blunt scalpel, tweezers, or a bone curette, firmly scrape the lesion, particularly at the advancing border. A bone curette is safe and useful for collecting specimens from babies, young children and

awkward sites such as interdigital spaces. If multiple lesions are present choose the most recent for scrapings as old loose scale is often not satisfactory. Any small vellus hairs when present within the lesions should be epilated. The tops of any fresh vesicles should be removed as the fungus is often plentiful in the roof of the vesicle.
In patients with suspected candidiasis the young "satellite" lesions which have not undergone exfoliation are more likely to yield positive results if they are present. Otherwise the advancing scaly border should be scraped. When lesions in the flexures are moist and very inflamed it is more satisfactory and less painful to roll a moistened swab firmly over the surface.
In patients with suspected cutaneous manifestations of systemic pathogens scrap the lesions with a bone curette or blunt scalpel as for tinea. A biopsy may be required in some cases.
NOTE: Following the collection of skin scales all scraped lesions should be firmly rubbed with a swab moistened in BHI broth.

Skin Scrapings, nail scrapings and epilated hairs where tinea is the provisional diagnosis:

1. Make a wet mount preparation in KOH for direct microscopy. Note a Calcofluor stained mount may also be necessary.
2. Inoculate specimen onto two slopes containing cycloheximide (actidione) i.e. one DERMASEL agar slope and one LACTRITMEL agar slope also containing chloramphenicol, gentamicin and incubate cultures at 26C. Maintain cultures for 4 weeks.
3. Where a moistened swab has also been collected from the same site as the scraping, inoculate this onto a Sabouraud's dextrose agar slope containing chloramphenicol and gentamicin, but NO cycloheximide and incubate at 26C. Maintain cultures for 4 weeks.

Skin scrapings and swabs where candidiasis is the provisional diagnosis:

A. Skin scrapings:

1. Make a wet mount preparation in KOH for direct microscopy. Note a Calcofluor stained mount may also be necessary.
2. Inoculate specimens onto Sabouraud's dextrose agar slopes containing chloramphenicol and gentamicin, but NO cycloheximide and incubate at 35C. Maintain cultures for 4 weeks.

B. Skin Swabs:

1. Smear swab onto heat sterilized glass slide for Gram stain.
2. Inoculate specimens onto Sabouraud's dextrose agar containing chloramphenicol and gentamicin, but NO cycloheximide and incubate at 35C. Maintain cultures for 4 weeks.
3. Where secondary bacterial infection is suspected, and separate swabs for routine bacteriology were not collected, the swab should first be inoculated onto a blood agar plate, followed by the Sabouraud's agar containing the antibiotics and then placed into Brain Heart Infusion Broth. All cultures should be incubated at 35C. Maintain cultures for 4 weeks.

NOTE: Where a dermatophyte is suspected or to be excluded a Sabouraud's agar slope containing cycloheximide and incubated at 26C may be included.

Scrapings from the groin, feet or nails where either a dermatophyte or Candida species may be isolated. This includes the possibility of a non-dermatophyte onychomycosis.

1. Direct Microscopy: Wet mount preparation in KOH for direct microscopy. Note a Calcofluor stained mount may also be necessary.
2. Inoculate specimens onto Sabouraud's dextrose agar containing chloramphenicol and gentamicin, but NO cycloheximide (as for Candida) and incubate at 26C. Maintain cultures for 4 weeks.
3. Inoculate specimen onto a DERMASEL agar slope containing cycloheximide (actidione), chloramphenicol and gentamicin and incubate cultures at 26C. Maintain cultures for 4 weeks.
4. Where a moistened swab has also been collected from the same site as the scraping, inoculate this onto a Sabouraud's dextrose agar slope containing chloramphenicol and gentamicin, but NO cycloheximide and incubate at 26C. Maintain cultures for 4 weeks.

Skin scrapings from patients with suspected pityriasis versicolor:

1. Direct Microscopy: Wet mount preparation in KOH for direct microscopy along with the cellotape stripping taken at the time of collection.
2. Inoculate scrapings onto an DIXON'S agar slope for isolation of Malassezia furfur and incubate cultures at 26C. Maintain cultures for 4 weeks.
3. Inoculate specimen onto Sabouraud's dextrose agar with chloramphenicol and gentainicin but NO cycloheximide (actidione) and incubate cultures at 26C. To exclude other yeasts like Candida. Maintain cultures for 4 weeks.
4. If dermatophytes are to be excluded also inoculate onto DERMASEL agar slope and incubate cultures at 26C. Maintain cultures for 4 weeks.

Skin scrapings from patients where a systemic pathogen is suspected:

1. Direct Microscopy: Wet mount preparation in KOH for direct microscopy. Note a Calcofluor stained mount may also be necessary.
2. Inoculate specimens onto:

(a) Sabouraud's dextrose agar with chloramphenicol and gentamicin but NO cycloheximide (actidione) and incubate duplicate cultures at 26C and 35C; and
(b) Brain heart infusion agar (BHIA) supplemented with 5% sheep blood and incubate at 35C. Maintain cultures for 4 weeks.

Sputum, Bronchial Washings and Throat Swabs

Many opportunistic mycoses have a pulmonary origin following the inhalation of fungal propagules. Bronchial washings and sputa should be collected upon rising in the morning as overnight incubation and growth of fungi in the lungs will increase the likelihood of isolating pathogenic fungi. Patients should not eat before specimen collection. Twenty-four hour samples are unacceptable because they become overgrown with bacteria and fungal contaminants. It should also be stressed that bronchial washings and sputa will usually be contaminated with throat flora. For this reason interpretation of results may be difficult from poor quality specimens.

Throat specimens are obtained by rolling a moist sterile swab over the affected area. However, if Candida is suspected the affected area will need to be scraped with a sterile tongue depressor.
All specimens must be sent to the laboratory and processed as soon as possible, a delay of longer than two hours at room temperature may impede the detection of some fungi. Store at 4C if short delays in processing are anticipated.
Unless it is already sufficiently fluid to pipette with a Pasteur pipette, sputa may need to be emulsified by shaking with 12-20 sterile glass beads and about 3-5ml of sterile distilled water, depending on the volume of the original specimen. Any bits of blood, pus or necrotic material should be plated directly onto media.
(1) Make wet mount preparations in KOH (1 drop) and Gram stained smears (1 drop) of all suspicious areas. The PAS stain may be necessary if the KOH preparation is unsatisfactory.
(2) Inoculate sample onto:
(a) Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate duplicate cultures at 26C and 35C; and
(b) Brain heart infusion agar (BHIA) supplemented with 5% sheep blood and incubate at 35C. Maintain cultures for 4 weeks.

Blood and Bone Marrow

The laboratory should be informed by the physician if fungal septicemia is suspected because special media are necessary for the optimum recovery of fungi. Numerous blood culture systems are available; however all systems must be vented to atmospheric air and incubated at 30C to maximize the rate and time of recovery of fungal organisms.
Aseptically collect 10 ml of blood and prepare several smears for Giemsa, Gram and PAS staining. Culture the remaining specimen by one or more of the following methods. With bone marrow aspirates the initial material is generally used for making smears for Giemsa staining, the remaining 3-5 ml of marrow and blood may be cultured on the media listed below.
1. Direct culture method: Inoculate 0.5-1.0 ml of buffy coat, prepared by centrifuging 5-10 ml of blood, onto the surface of the media listed below. This inoculum can then be spread over the surface of the agar with a sterile inoculating loop and the plate incubated aerobically at 30C. This method is suitable for small low volume laboratories where there are few requests for fungal blood cultures. Cultures should be maintained for 4 weeks.
2. Biphasic culture bottle: The recovery of fungi from blood may be enhanced by using a biphasic bottle containing a slant of brain heart infusion agar and 60-100 ml of BHI broth. A ratio of 1:10 to 1:20 (blood to broth) is recommended, a minimum of 5.0 ml of blood is required for each culture bottle. The biphasic culture bottle is kept vented and is tilted daily to allow broth to flow over the agar surface. These cultures must be carefully checked daily for growth. Because fungi will not turn the broth very cloudy it is imperative to frequently Gram stain the bottle contents to detect fungal elements. Cultures should be incubated at 30C and maintained for 4 weeks.

3. Membrane filter technique: This is a superior technique to the vented biphasic blood bottle used to concentrate and culture specimens of blood and CSF. Briefly, specimens are treated sequentially with Triton-X and sodium carbonate solutions to lyse blood cells and then filtered by vacuum through a 0.45 um membrane. This membrane is then placed onto the media listed below.

4. Lysis centrifugation isolator system: The Wampole Isolator system has been found to significantly improve the recovery of fungi from blood and is strongly recommend by Koneman and Roberts (1985) as the method of choice for processing blood cultures from patients with suspected fungal septicemia. The Isolator utilizes a tube that contains components that lyse leukocytes and erythrocytes and also inactivate plasma complement and certain antibiotics. Once lysed, the cells release the microorganisms contained within them, and the centrifugation step in the procedure serves to concentrate the organisms in the blood sample. This concentrate is then inoculated onto the surface of appropriate culture media listed below. Ten milliliters of blood are required for each tube and cultures should be incubated at 30oC and maintained for 4 weeks.

5. Bactec: Bactec have produced a special fungal media (BACTEC Fungal Medium) for enhanced fungal blood culture using their non-radiometric (NR) instruments. Once again, blood cells are lysed by the medium to enhance recovery of fungi. Note antimicrobials have also been added to limit the growth of bacteria.

Primary isolation media for blood and bone marrow culture: (a) Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate duplicate cultures at 26oC and 35oC; and (b) Brain heart infusion agar (BHIA) supplemented with 5% sheep blood and incubate at 35C. Maintain cultures for 4 weeks.

Note: Negative bacteriological cultures from patients with clinical evidence of an infection should be sealed with tape and maintained at 26C for 4 weeks to exclude the presence of a slow growing fungus

Urine should be collected first thing in the morning after overnight incubation in the bladder. A midstream clean catch or catheterized specimen is best, as this minimizes the presence of genital flora. Do not use urine from a collection bag or bed pan. Twenty-four hour urine samples are unacceptable.

Yeasts recovered from routine urine bacteriology cultures of catheterized urine or urine obtained by sterile procedure should be identified and reported regardless of colony count. However, the isolation of yeasts from clean catch specimens must be interpreted with caution and is not significant without additional support from other clinical and laboratory investigations.

Note: Negative bacteriological cultures from patients with clinical evidence of an infection should be sealed with tape and maintained at 26C for 4 weeks to exclude the presence of a slow growing fungus.

Urine samples must be processed as soon as possible, a delay of longer than two hours at room temperature may impede the detection of some fungi. Store at 4oC if short delays in processing are anticipated.

(1) Centrifuge the urine for 10-15 minutes at 2000 rpm. Decant the supernatant and pool the sediment if necessary.
(2) Prepare a direct smear of the sediment in KOH for direct microscopy. Note PAS, Gram or India ink preparations may also be helpful.
(3) Inoculate 0.05-0.1 ml of the sediment onto Sabouraud's agar with gentamicin and chloramphenicol and incubate duplicate cultures at 26C & 35C. Maintain cultures for 4 weeks.

Cerebrospinal Fluid (CSF):

Three to five milliliters of CFS is optimal for fungal investigation, however lesser volumes are often received and should be processed. CSF specimens should be transported to the laboratory as soon as possible and processed promptly. If there is a delay do not refrigerate the samples, rather they should be left at room temperature or incubated at 30C. The specimen should be centrifuged. Keep the supernatant for cryptococcal antigen testing and process the sediment as follows.
(1) For direct microscopy use 1 drop of the sediment to make an India ink mount.
(2) Resuspend the remaining sediment in 1-2 ml of CSF and inoculate onto; (a) Sabouraud's dextrose agar with chloramphenicol and gentamicin and incubate duplicate cultures at 26C and 35C; and (b) Brain heart infusion agar (BHIA) supplemented with 5% sheep blood and incubate at 35C. Maintain cultures for at least 4 weeks.
Note: Cultures from patients undergoing treatment for cryptococcal meningitis should be maintained for 3 months, so that dormant viable cells, which do not start to grow until after a one month period, will not be missed.

Yeasts Identifications

Blastoschizomyces
Candida
Cryptococcus
Loboaloboa
Malassezia
Rhodotorula
Saccharomyces
Trichosporon

Identification.

Yeast-like fungi may be basidiomycetes, such as *Cryptococcus neoformans* or ascomycetes such as *Candida albicans*.

1. Ensure that you start with a pure culture; streak for single colony isolation if necessary.

2. Germ Tube Test: lightly inoculated 5 ml of serum, containing 0.5% glucose and incubated at 35oC for 2-3 hours.
Positive = *Candida albicans* or *Candida dubliniensis*.
Negative or from HIV positive patient = perform assimilation tests.

3. For the identification of germ tube negative yeasts, morphological (Dalmau plate culture), physiological and biochemical tests are essential.

Dalmau Plate Culture: To set up a yeast morphology plate, dip a flamed sterilized straight wire into a light inoculum (sterile distilled water suspension) and then lightly scratch the wire into the surface of a cornmeal/tween 80, rice/tween 80 or yeast morphology agar plate, then place a flamed coverslip onto the agar surface covering the scratches. Dalmau morphology plates are examined in situ using the lower power of a microscope for the presence of pseudohyphae which may take up to 4-5 days at 26oC to develop. *C. albicans* also produces characteristic large, round, terminal, thick-walled vesicles (often called chlamydoconidia). The key features to remember are to use a light inoculum and to scratch the surface of the agar with the wire when inoculating.

Physiological and biochemical tests: including fermentation and assimilation studies should be performed. Reliable commercially available yeast identification kits are the API 20C AUX, ATB 32C, MicroScan and Vitek systems

Identification of Common Dermatophytes.

Microscopic morphology of the micro and/or macroconidia is the most reliable identification character, but you need a good slide preparation and you may need to stimulate sporulation in some strains. Culture characteristics such as surface texture, topography and pigmentation are variable and are therefore the least reliable criteria for identification. Clinical information such as the site, appearance of the lesion, geographic location, travel history, animal contacts and race is also important, especially in identifying rare non-sporulation species like *M. audouini*, *T. concentricum* and *T schoenleinii* etc. Note: mating experiments are not practical for the clinical mycology laboratory.

Three genera are recognized:

Epidermophyton:

Smooth thin-walled Macroconidia only present, no microconidia, colonies a green-brown to khaki colour.

Microsporum:

Macroconidia with rough walls present, microconidia may also be present.

Trichophyton:

Microconidia present, smooth-walled macroconidia may or may not be present.

Lactophenol Cotton Blue.

For the staining and microscopic identification of fungi

Cotton Blue (Aniline Blue)	0.05 g
Phenol Crystals (C6H5O4)	20 g
Glycerol	40 ml
Lactic acid (CH3CHOH COOH)	20 ml
Distilled water	20 ml

Method

This stain is prepared over two days.

1. On the first day, dissolve the Cotton Blue in the distilled water. Leave overnight to eliminate insoluble dye.
2. On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker. place on magnetic stirrer until the phenol is dissolved.

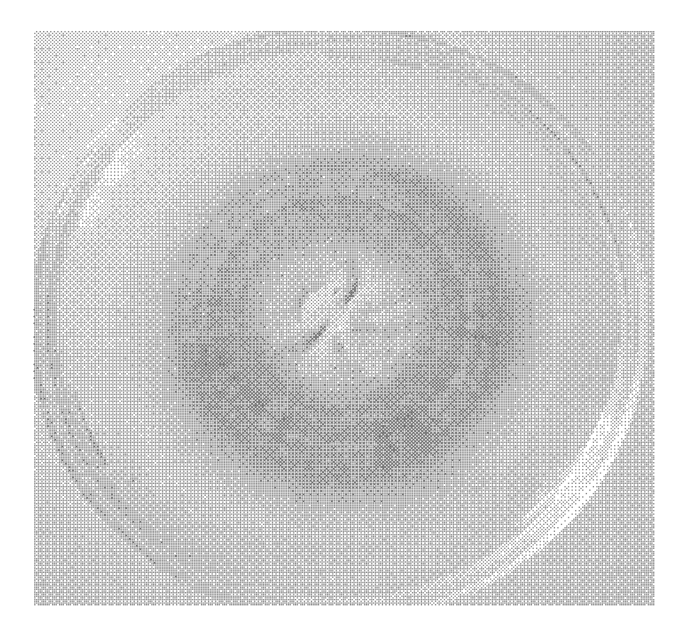
3. Add the glycerol.
4. Filter the Cotton Blue and distilled water solution into the phenol/glycerol/lactic acid solution. Mix and store at room temperature.
Sabouraud's Dextrose Agar for Dermatophytes
Sabouraud's Dextrose Agar with Cycloheximide, Chloramphenicol, Gentamicin and Yeast Extract.

For the primary isolation and cultivation of dermatophytes.

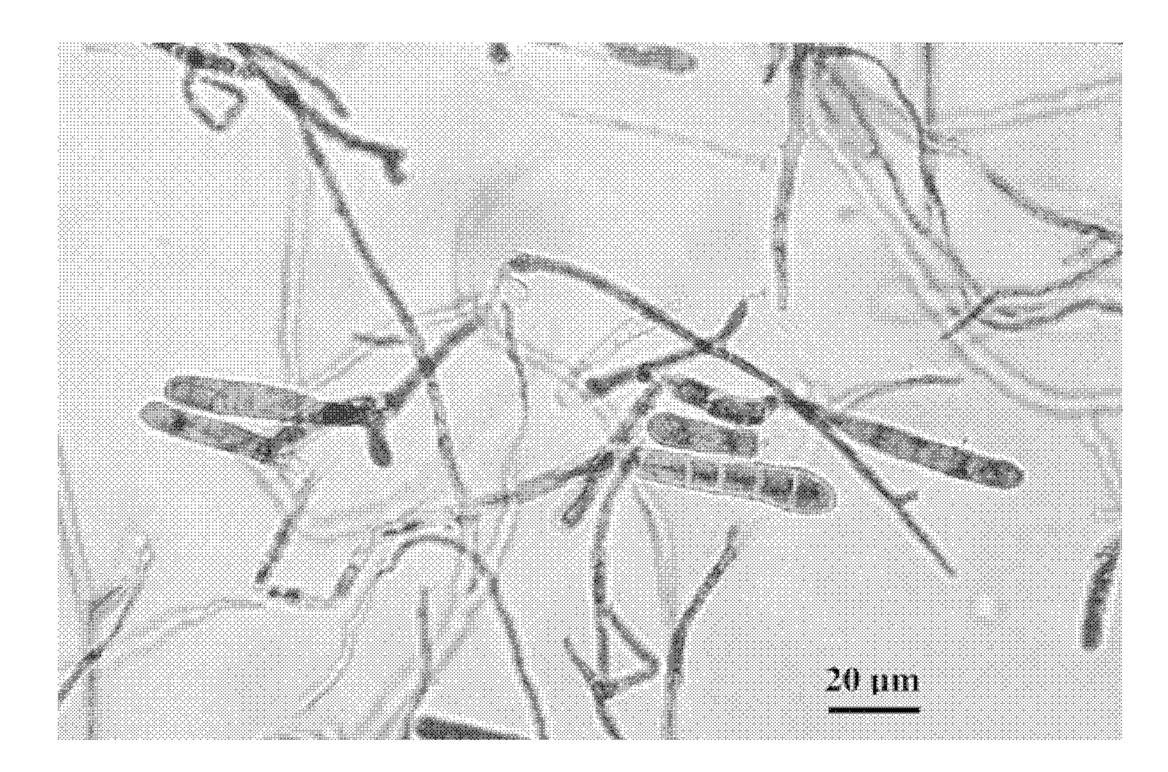
Sabouraud's Dextrose Agar (Oxoid CM41)	**65 g**
Cycloheximide (Actidione)	**0.5 g**
Chloramphenicol	**1x250 mg capsule**
Yeast extract	**5 g**
Gentamicin (40 mg/ml)	**0.65 ml**
Distilled water	**1000 ml**

Method:
1. Soak all ingredients, except Gentamicin, in 100 ml water.
2. Boil remaining water, add to soaking ingredients, and bring to boil to dissolve, stirring well to prevent from charring.
3. Add the Gentamicin. Mix well.
4. Dispense for slopes if required.
5. Autoclave at 121C for 10 minutes. Remove and slope, or pour for plates as required.

Epidemophyton floccosum



E. floccosum on mycobiotic agar. Colonies are usually greenish-brown or khaki coloured with a suede-like surface, raised and folded in the centre, with a flat periphery and submerged fringe of growth. Older cultures may develop white pleomorphic tufts of mycelium. A deep yellowish-brown reverse pigment is usually present.



Epidemophyton floccosum

Microscopic morphology of *E. floccosum* showing characteristic smooth, thin-walled macroconidia, which are often produced in, clusters growing directly from the hyphae.

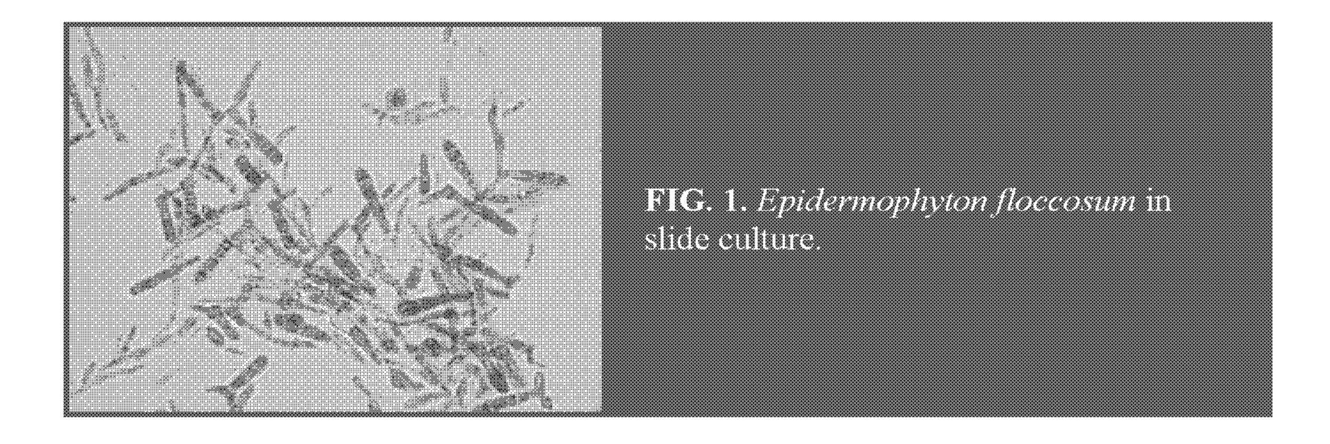
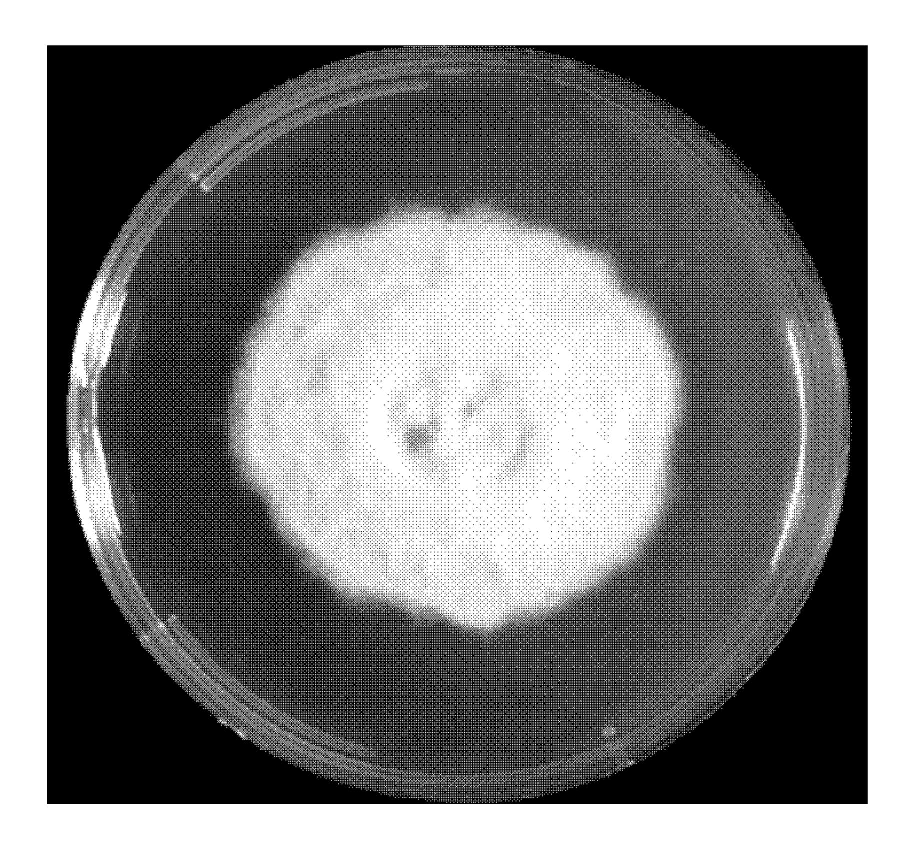


FIG. 1. *Epidermophyton floccosum* in slide culture.



Microsporum audouinii

Colony of *M. audouinii* on mycobiotic agar showing a flat, spreading, downy white surface with a salmon or peach-pink reverse pigment.

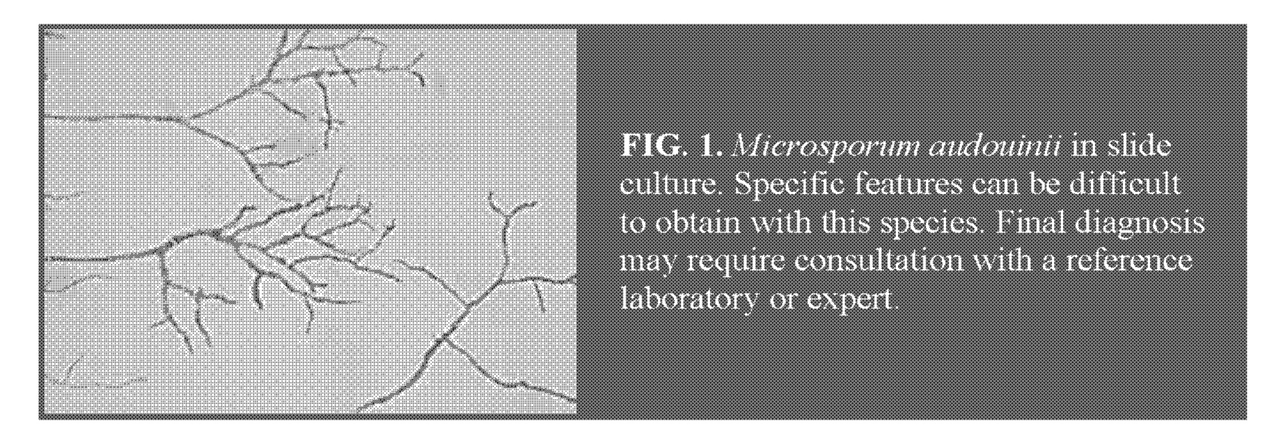
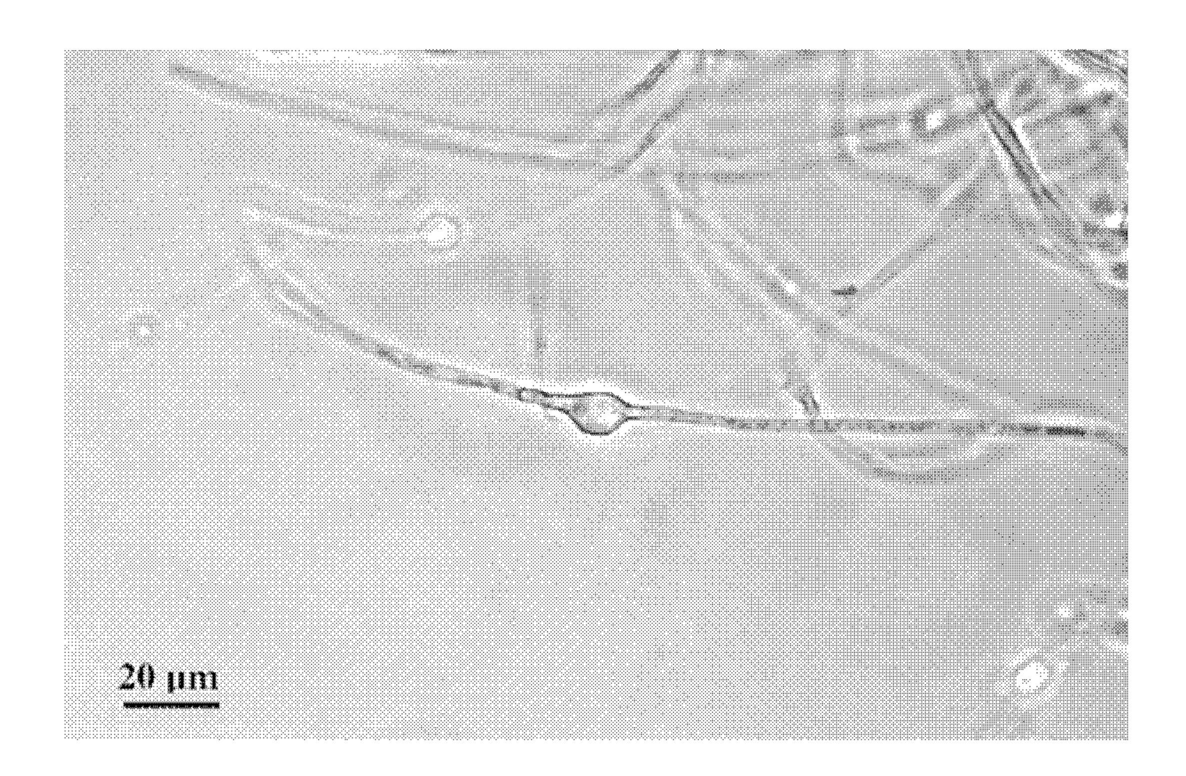
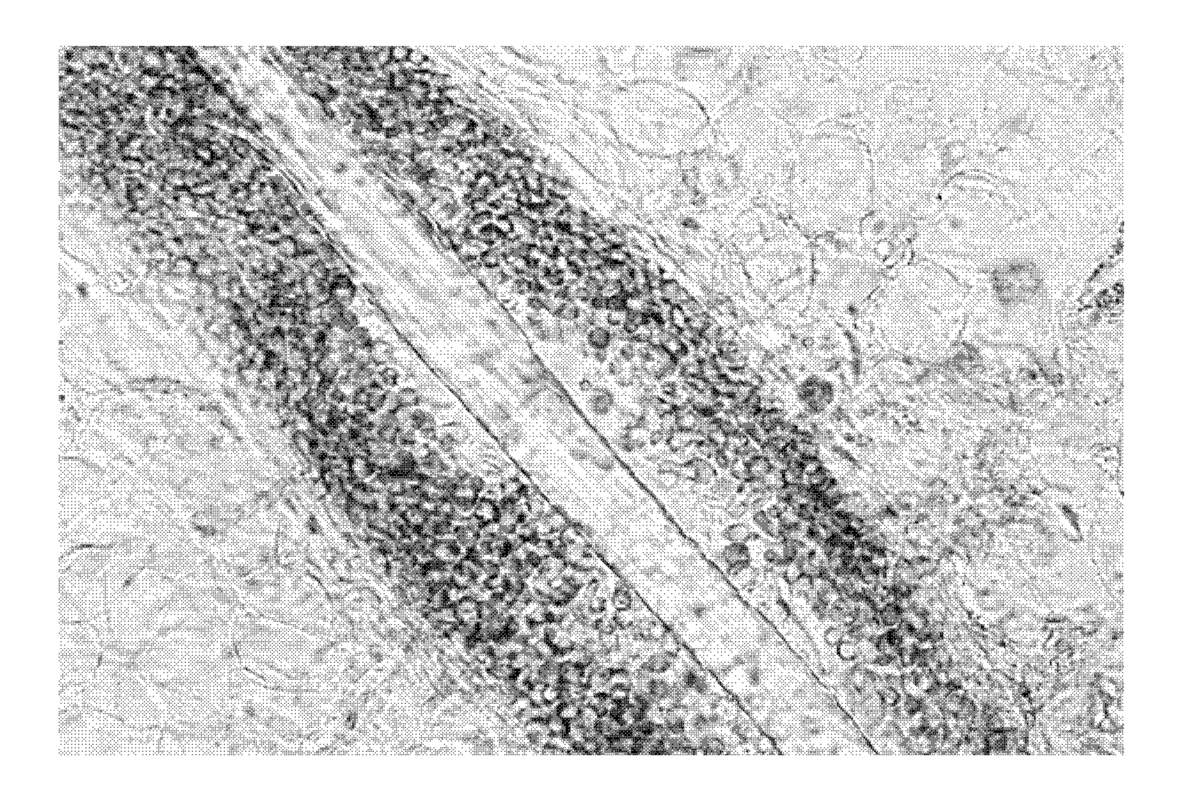


FIG. 1. *Microsporum audouinii* in slide culture. Specific features can be difficult to obtain with this species. Final diagnosis may require consultation with a reference laboratory or expert.



Microsporum audouinii

Microscopy morphology of *M. audouinii* showing a thick-walled intercalary chlamydoconidium. Note macroconidia and microconidia are only rarely produced.



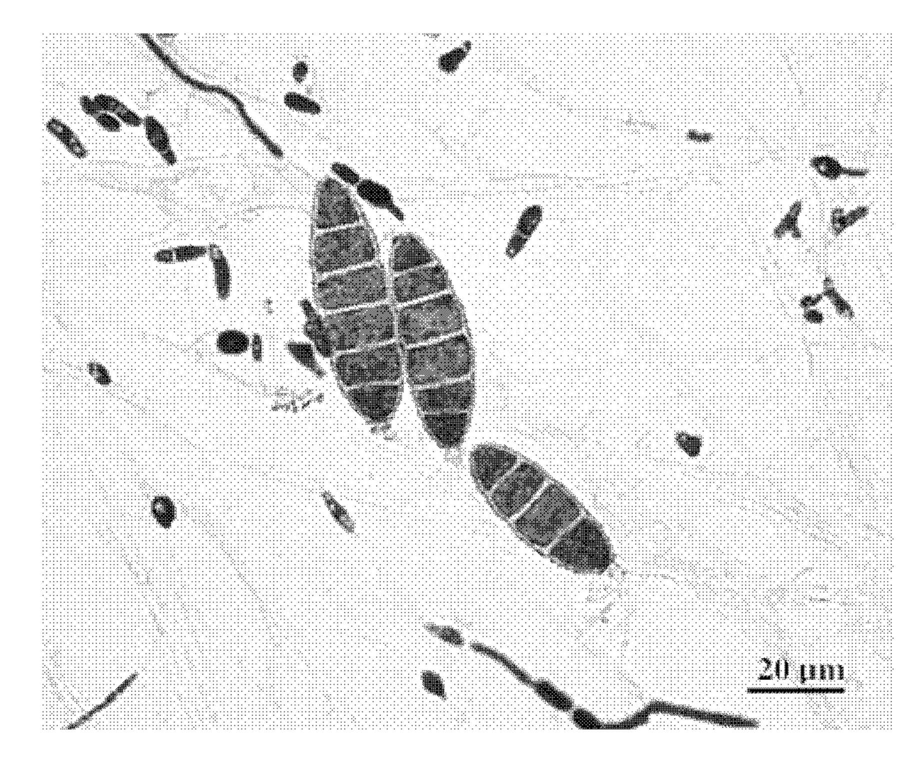
Microsporum gypseum

Scalp infection in a young child showing hair loss and a circular erythematous kerion-like lesion. Note small broken-off infected hairs. *M. gypseum* was isolated.



Microsporum gypseum

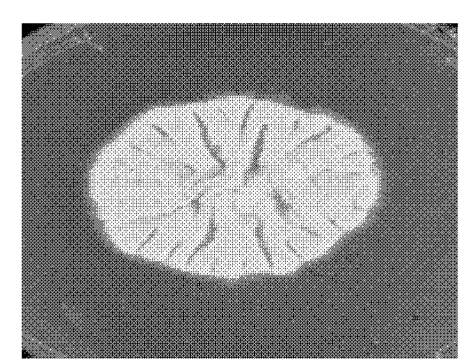
Culture of *M. gypseum* on mycobiotic agar showing a flat, spreading suede-like to granular, tawny-buff cinnamon coloured surface. Many cultures develop a central white downy umbo (dome) or a fluffy white tuft of mycelium and some also show a narrow, white periphery. A yellow-brown pigment is usually produced on the reverse, however a reddish-brown reverse pigment may be present in some strains.



Microsporum gypseum

Microscopic morphology *M. gypseum* showing abundant, symmetrical, ellipsoidal, thin-walled verrucose 4-6 celled macroconidia. The terminal or distal ends of most macroconidia are slightly rounded, while the proximal ends (point of attachment to hyphae) are truncate. Numerous clavate shaped microconidia are also present, but these are not diagnostic.

Trichophyton verrucosum

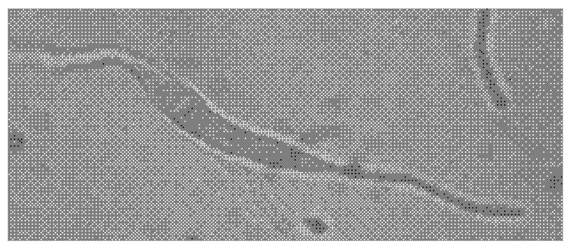


culture of *Trichophyton verrucosum*.

On Sabouraud's dextrose agar, colonies are slow growing, small, button-or-disk-shaped, white to cream coloured, with a suede-like to velvety surface, a raised centre, and flat periphery with some submerged growth. Reverse pigment may vary from non-pigmented to yellow. Broad, irregular hyphae with many terminal and intercalary chlamydospores. Chlamydospores are often in chains. The tips of some hyphae are broad and club-shaped, and occasionally divided, giving the so-called "antler" effect. When grown on thiamine-enriched media, occasional strains produce clavate to pyriform microconidia borne singly along the hyphae. Macroconidia are only rarely produced, but when present have a characteristic tail or string bean shape.



Clavate to pyriform microconidia of *T. verrucosum*.

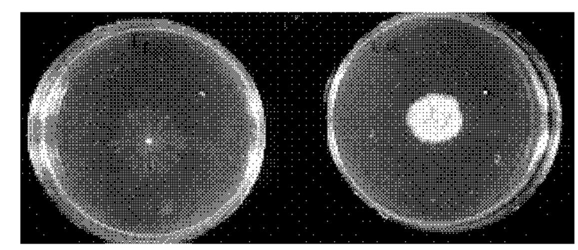


Characteristic rat tail or string bean shaped macroconidia of *T. verrucosum.*

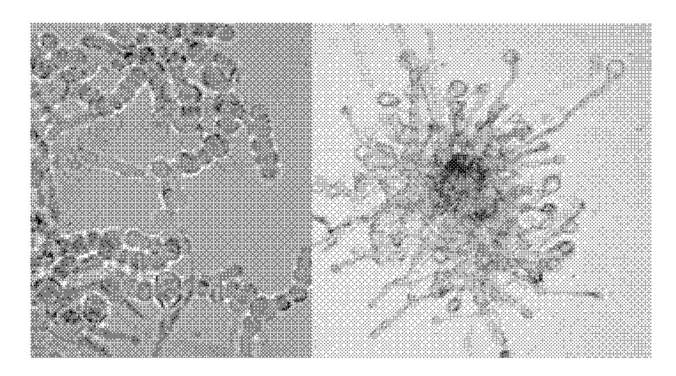
Confirmatory tests:

Growth at 37C: unlike other dermatophytes growth is enhanced at 37C

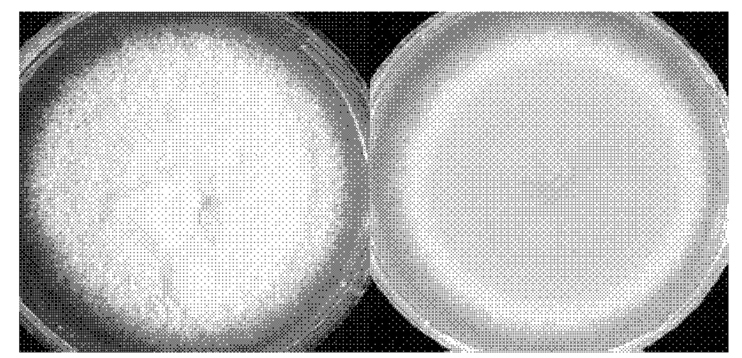
Nutritional requirements: all strains require thiamine and approximately 80% require thiamine and inositol. There is no growth on casein vitamin free agar (T1), minimal submerged growth on T1 + inositol (T2), good growth on T1 + inositol and thiamine (T3) and no growth on T1 + thiamine only (T4).



Growth on T1 vitamin free agar vs T3 with inositol and thiamine.

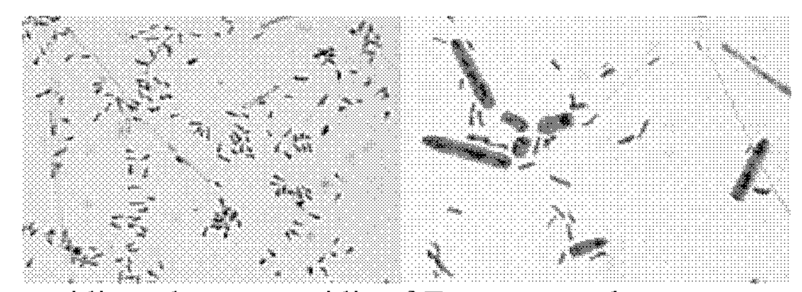


Trichophyton mentagrophytes var. *erinacei*



Culture of *T. mentagrophytes* var. *erinacei* showing its typical brilliant lemon yellow reverse pigment.

On Sabouraud's dextrose agar, colonies are white, flat, powdery, sometimes downy to fluffy with a brilliant lemon yellow reverse. Numerous large clavate microconidia are borne on the sides of hyphae. Macroconidia are smooth, clavate, variable in size, and may have terminal appendages.



Microconidia and macroconidia of *T. mentagrophytes* var. *erinacei* .

Kaminski's Dermatophyte Identification Scheme:

Littman Oxgall Agar (Difco): White, downy colony with yellowish green diffusible pigment.

Lactritmel Agar (Mycopathologia 91:57-59, 1985): White suede to powdery colony with brilliant yellow reverse. Numerous large slender clavate microconidia.

Sabouraud's Dextrose Agar with 5% NaCl: White folded suede to powdery colony with no reverse pigment.

1% Peptone Agar: White suede to powdery colony with pale yellow reverse.

Trichophyton Agar No. l: Good growth indicating no special nutritional requirements. Colonies are white suede to powdery with no reverse pigment.

Hydrolysis of Urea: Negative at 7 days

Vitamin Free agar (Difco Trichophyton Agar No.1): Good growth indicating no special nutritional requirements. Cultures are white, folded, suede-like to powdery with no reverse pigment.

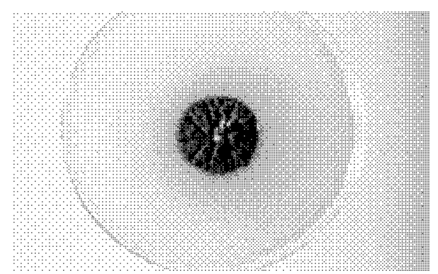
Hair Perforation Test ("in vitro"): Positive

T. mentagrophytes var. *erinacei* is generally distinguished from other varieties of *T. mentagrophytes* by (a) its microscopic morphology showing numerous large slender clavate microconidia borne on the slides of hyphae and its smooth, thin-walled clavate macroconidia; (b) its brilliant lemon yellow reverse pigment on plain Sabouraud's agar and Lactritmel agar; (c) its lack of reverse pigment on Sabouraud's salt agar; and (d) its negative hydrolysis of urea.

Clinical significance:

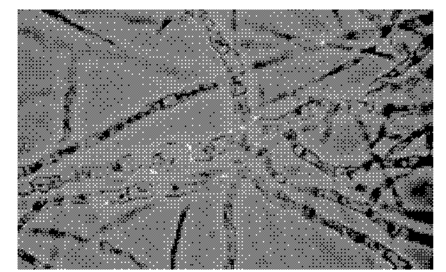
Trichophyton mentagrophytes var. erinacei is a zoophilic fungus associated with hedgehogs and the epidermal mites which they often harbour. Human infections occur most frequently on the exposed parts of the body; but tinea of the scalp and nails can also occur. Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultra-violet light. The distribution of this fungus is New Zealand and Europe.

Trichophyton violaceum



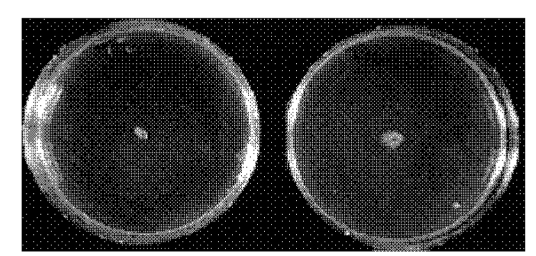
Culture of *Trichophyton violaceum.*

On Sabouraud's dextrose agar, colonies are very slow growing, glabrous or waxy, heaped and folded and a deep violet in colour. Cultures often become pleomorphic, forming white sectors and occasional non-pigmented strains may occur. Hyphae are relatively broad, tortuous, much branched and distorted. Young hyphae usually stain well in lactophenol cotton blue, whereas older hyphae stain poorly and show small central fat globules and granules. No conidia are usually seen, although occasional pyriform microconidia have been observed on enriched media. Numerous chlamydoconidia are usually present, especially in older cultures.



Chlamydoconidia of *T. violaceum*.

Nutritional requirements: T. violaceum has a partial nutrient requirement for thiamine. There is minimal growth on casein vitamin-free agar (Trichophyton Agar No. 1), and slightly better growth on vitamin-free agar plus thiamine (Trichophyton Agar No. 4). The partial requirement for thiamine separates this organism from *T. gourvillii, T. rubrum*, and other species that may produce purple pigmented colonies.

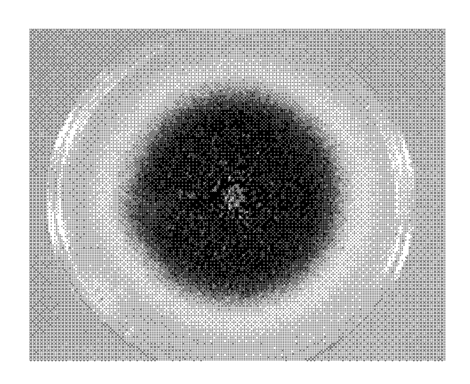


T. violaceum growth on T4 agar showing a partial requiremnet for thiamine.

Clinical significance:

Trichophyton violaceum is an anthropophilic fungus causing inflammatory or chronic non-inflammatory finely scaling lesions of skin, nails, beard and scalp, producing the so-called "black dot" tinea capitis. Distribution is world-wide, particularly in the Near East, Eastern Europe, USSR and North Africa. Invaded hairs show an endothrix infection and do not fluoresce under Wood's ultra-violet light.

Aspergillus fumigatus



Culture of *Aspergillus fumigatus.*

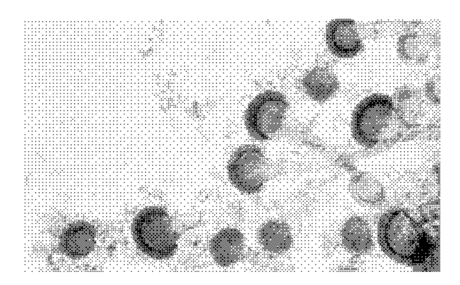
On Czapek dox agar, colonies show typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores.

Conidial heads are typically columnar (up to 400 x 50 um but often much shorter and smaller) and uniseriate.

Conidiophores are short, smooth-walled and have conical-shaped terminal vesicles which support a single row of phialides on the upper two thirds of the vesicle.

Conidia are produced in basipetal succession forming long chains and are globose to subglobose (2.5-3.0 um in diameter), green and rough-walled.

Note, this species is thermotolerant and grows at temperatures up to 55C.



Conidial head of *A. fumigatus*
(Note: uniseriate row of phialides on the upper two thirds of the vesicle).

Clinical significance:

Aspergillus fumigatus is truly a cosmopolitan mould and has been found almost everywhere on every conceivable type of substrate, especially soil and decaying organic debris.

A. fumigatus is an important human pathogen and it is the most common cause of all forms of invasive and non-invasive aspergillosis

Laboratory Diagnosis of Viral Diseases

I. If you can't treat viral diseases, why diagnose them?
a. some are amenable to chemotherapy
b. determines how patient will be treated - particularly in trying to avoid congenital defects (CMV)
c. public health measures to prevent spread - quarantine, sequestration, vector control
d. surveillance - new strains, new diseases, new viruses, new virus-disease associations, efficacy of immunization procedures

Proper viral diagnosis requires appropriate collection of specimens

Site of collection is dictated by symptomology, but as general rule the epithelial surface that is the port of entry and the primary site of viral replication should be sampled. So for respiratory infections, it would be a throat or nasal swab. For enteric infection - feces. For possible meningitis - CSF. Because viruses tend to be very labile they are put into a buffered salt solution (with gelatin added for stabilization) and kept at 4°C for transport (if the specimen needs to be shipped).

Overview of Diagnostic Methods

In general, diagnostic tests can be grouped into 3 categories.:

1. Direct Examination of Specimen

1. Electron Microscopy morphology / immune electron microscopy
2. Light microscopy histological appearance - e.g. inclusion bodies
3. Antigen detection immunofluorescence, ELISA etc.
4. Molecular techniques for the direct detection of viral genomes

2. Indirect Examination

1. Cell Culture - cytopathic effect, haemadsorption, confirmation by neutralization, interference, immunofluorescence etc.
2. Eggs pocks on CAM - haemagglutination, inclusion bodies
3. Animals disease or death confirmation by neutralization

3. Serology

Detection of rising titres of antibody between acute and convalescent stages of infection, or the detection of IgM in primary infection.

In direct examination, the clinical specimen is examined directly for the presence of virus particles, virus antigen or viral nucleic acids.

In indirect examination, the specimen into cell culture, eggs or animals in an attempt to grow the virus: this is called virus isolation.

Serology actually constitute by far the bulk of the work of any virology laboratory. A serological diagnosis can be made by the detection of rising titres of antibody between acute and convalescent stages of infection, or the detection of IgM. In general, the majority of common viral infections can be diagnosed by serology.

The specimen used for direction detection and virus isolation is very important. A positive result from the site of disease would be of much greater diagnostic significance than those from other sites. For example, in the case of herpes simplex encephalitis, a positive result from the CSF or the brain would be much greater significance than a positive result from an oral ulcer, since reactivation of oral herpes is common during times of stress.

Classical Techniques	**Newer Techniques**
1. Complement fixation tests (CFT)	1. Radioimmunoassay (RIA)
2. Haemagglutination inhibition tests	2. Enzyme linked immunosorbent assay (EIA)
3. Immunofluorescence techniques (IF)	3. Particle agglutination
4. Neutralization tests	4. Western Blot (WB)
5. Single Radial Haemolysis	5. Recombinant immunoblot assay (RIBA), line immunoassay (Liatek) etc.

<u>Virus Isolation</u>

Viruses are obligate intracellular parasites that require living cells in order to replicate. Cultured cells, eggs and laboratory animals may be used for virus isolation. Although embroyonated eggs and laboratory animals are very useful for the isolation of certain viruses, cell cultures are the sole system for virus isolation in most laboratories. The development of methods for cultivating animal cells has been essential to the progress of animal virology. To prepare cell cultures, tissue fragments are first dissociated, usually with the aid of trypsin or collagenase. The cell suspension is then placed in a flat-bottomed glass or plastic container (petri dish, a flask, a bottle, test tube) together with a suitable liquid medium. e.g. Eagle's, and an animal serum. After a variable lag, the cells will attach and spread on the bottom of the container and then start dividing, giving rise to a primary culture. Attachment to a solid support is essential for the growth of normal cells.

Primary and Secondary Cultures

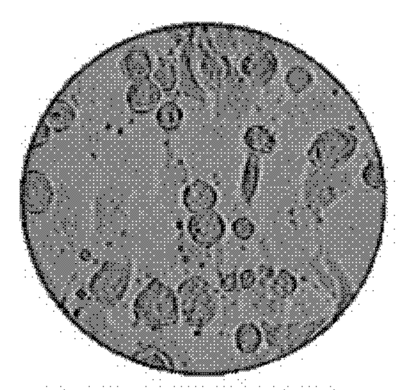
Primary cultures are maintained by changing the fluid 2 or 3 times a week. When the cultures become too crowded, the cells are detached from the vessel wall by either trypsin or EDTA, and portions are used to initiate secondary cultures. In both primary and secondary cultures, the cells retain some of the characteristics of the tissue from which they are derived.

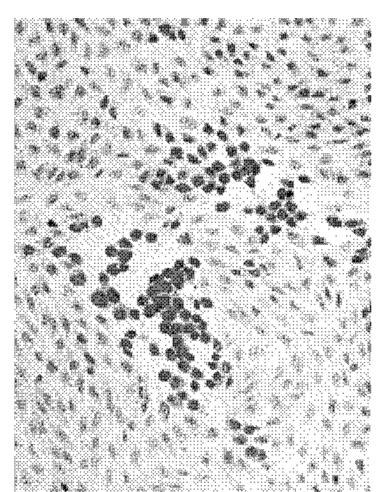
Cell Strains and Cell Lines
Cells from primary cultures can often be transferred serially a number of times. The cells may then continue to multiply at a constant rate over many successive transfers. Eventually, after a number of transfers, the cells undergo culture senescence and cannot be transferred any longer. For human diploid cell cultures, the growth rate declines after about 50 duplications. During the multiplication of the cell strain, some cells become altered in that they acquire a different morphology, grow faster, and become able to start a cell culture from a smaller number of cells. These cells are immortalized and have an unlimited life-span. However, they retain contact inhibition.
Cell Cultures
Cell cultures are separated into 3 types:-
1. **Primary cells** - prepared directly from animal or human tissues and can be subcultured only once or twice e.g. primary monkey or baboon kidney
2. **Semi-continuous diploid cells** - which are derived from human fetal tissue and can be subcultured 20 to 50 times e.g. human diploid fibroblasts such as MRC-5
3. **Continuous cells** - derived from tumours of human or animal tissue e.g. Vero, Hep2

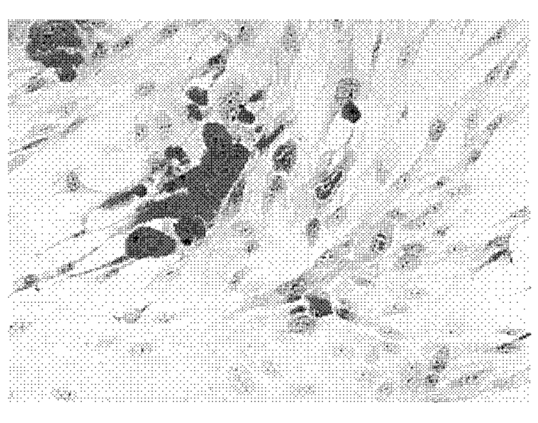
Cell cultures vary greatly in their susceptibility to different viruses. It is of utmost importance that the most sensitive cell cultures are used for a particular suspected virus. Specimens for cell culture should be transported to the laboratory as soon as possible upon being taken. Swabs should be put in a vial containing virus transport medium. Bodily fluids and tissues should be placed in a sterile container.

Upon receipt, the specimen is inoculated into several different types of cell culture depending on the nature of the specimen and the clinical presentation. The maintenance media should be changed after 1 hour or if that is not practicable, the next morning. The inoculated tubes should be incubated at 35-37oC in a rotating drum. Rotation is optimal for the isolation of respiratory viruses and result in an earlier appearance of the CPE for many viruses. If stationary tubes are used, it is critical that the culture tubes be positioned so that the cell monolayer is bathed in nutrient medium.

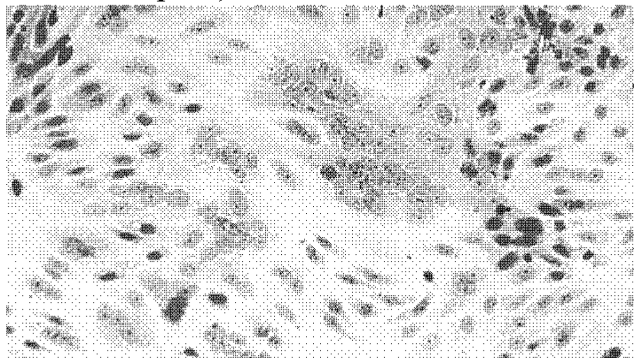
The inoculated tubes should be read at least every other day for the presence of cytopathic effect. Certain specimens, such as urine and faeces, may be toxic to cell cultures that may produce a CPE-like effect. If toxic effects are extensive, it may be necessary to passage the inoculated cells. Cell cultures that are contaminated by bacteria should either be put up again or passed through a bacterial filter. Cell cultures should be kept for at least one to two weeks (longer in the case of CMV). Cell cultures should be refed with fresh maintenance medium at regular intervals or if required should the culture medium become too acidic or alkaline. When CPE is seen, it may be advisable to passage infected culture fluid into a fresh culture of the same cell type. For cell-associated viruses such as CMV and VZV, it is necessary to trypsinize and passage intact infected cells. Other viruses such as adenovirus can be subcultured after freezing and thawing infected cells.

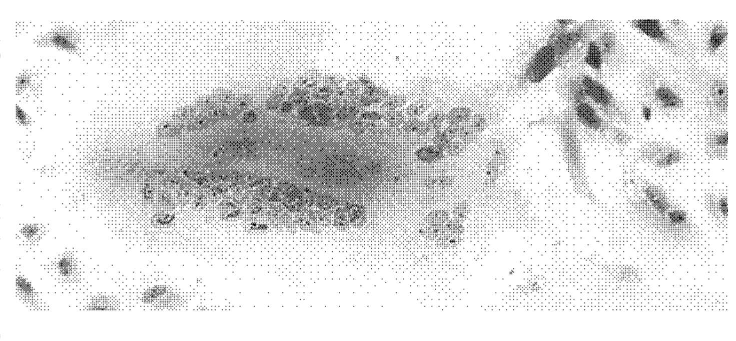






Cytopathic effects of enterovirus 71, HSV, and CMV in cell culture: note the ballooning of cells. (Linda Stannard, University of Cape Town, Virology Laboratory, Yale-New Haven Hospital)





Cytopathic effects of mumps and measles viruses in cell culture: note the formation of syncytia. (Courtesy of Linda Stannard, University of Cape Town, S.A.)

Influenza and parainfluenza viruses do not ordinarily induce CPE, however they possess haemagglutinins and thus the ability to absorb guinea pig RBCs as they bud from the cell. This phenomenon is known as haemadsorption. Commonly employed cell cultures include primary monkey kidney, LLC-MK2 and MDCK cells. The cell cultures are incubated with a suspension of guinea pig RBCs at 4°C or RT for 30 minutes. The unabsorbed RBCs are then removed and the cell sheet observed microscopically for the presence of haemadsorption. Presumptive identification of virus isolates can usually be made on the basis of the type of CPE, haemadsorption, and selective cell culture susceptibility. For final identification, immunofluorescence, neutralization, haemadsorption inhibition, lectron microscopy, or molecular tests are normally carried out.

Advantages of cell culture for virus diagnosis include relative ease, broad spectrum and sensitivity.

It is limited by the difficulty in maintaining cell cultures, variability of cell cultures. Contamination by endogenous viral agents such as SV40, mycoplasma and bacteria may occur. Another problem in isolating certain viruses, especially myxo and paramyxo viruses is the presence of inhibitory substances or antibodies in the calf serum used in the cell culture media. Using fetal calf serum reduces this problem but adds to the expense.

Rapid Culture Techniques e.g. DEAFF test

One of the most significant contributions to rapid diagnosis has been the application of centrifugation cultures to viral diagnosis. For a number of years, it has been recognized that low-speed centrifugation of specimens onto cell monolayers enhances the infectivity of certain viruses as well as chlamydia. The cell culture is stained by monoclonal antibodies for the presence of specific viral antigens 24-48 hours later. The best known example of this technique is the DEAFF test used for the early diagnosis of CMV infection. In the DEAFF test, the specimen is inoculated onto human embroyonic fibroblasts and then spun at a low speed. After a period of 24-48 hours, the cells are then stained by monoclonal antibodies against CMV early antigen. Therefore a rapid diagnosis of CMV infection can be made without having to wait 1-3 weeks for the CPE to appear.

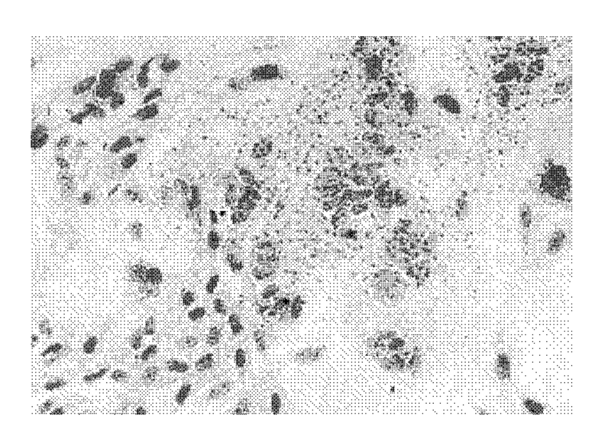




Fig. 2, CMV centrifugation culture fixed and stained 16 hrs after inoculation showing viral proteins in nuclei of infected human fibroblast cells

Left: Haemadsorption of red blood cells onto the surface of a cell sheet infected by mumps virus. Also note the presence of syncytia which is indistinguishable from that of RSV (Courtesy of Linda Stannard, University of Cape Town). Right: Positive CMV DEAFF test. (Virology Laboratory, Yale-New Haven Hospital)

Susceptible Cell Lines

1. **Herpes Simplex** Vero Hep-2, human diploid (HEK and HEL),human amnion
2. **VZV** human diploid (HEL, HEK)
3. **CMV** human diploid fibroblasts
4. **Adenovirus** Hep2, HEK,
5. **Poliovirus** MK, BGM, LLC-MK2, human diploid, Vero, Hep-2,Rhadomyosarcoma
6. **Coxsackie B** MK, BGM, LLC-MK2, vero, hep-2
7. **Echo** MK, BGM, LLC-MK2, human diploid, Rd

8. **Influenza A** MK, LLC-MK2, MDCK
9. **Influenza B** MK, LLC-MK2, MDCK
10. **Parainfluenza** MK, LLC-MK2
11. **Mumps** MK, LLC-MK2, HEK, Vero
12. **RSV** Hep-2, Vero
13. **Rhinovirus** human diploid (HEK, HEL)
14. **Measles** MK, HEK
15. **Rubella** Vero, RK13

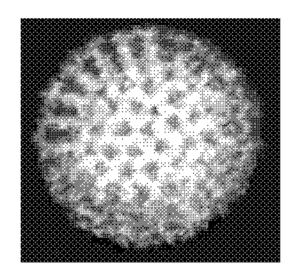
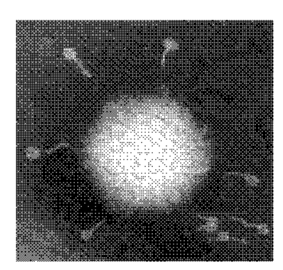
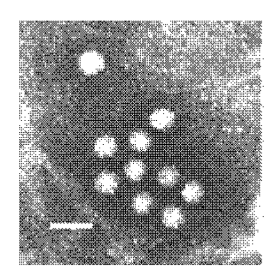
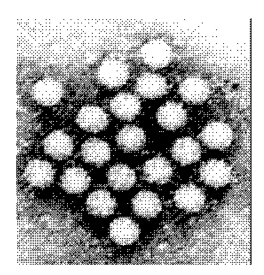
2.) Electron Microscopy

Virus diagnosis by electron microscopy relies on the detection and identification of viruses on the basis of their characteristic morphology. A major advantage of virus diagnosis by EM is the ability to visualize the virus. By identifying the virus directly, it is possible to perform an examination without a preconceived concept of the aetiological agent, in contrast with those assays which require a specific viral probe. Speed is another advantage of EM as the specimen can be processed within minutes of receipt and thus EM can be used as a rapid diagnostic method. On the other hand, the main disadvantage of EM is its inability to examine multiple specimens coincidentally. Secondly, there must be a minimum number of virus particles present (around 10^6 virus particles per ml for detection) Some viruses such as SRSV may give a non-distinct morphological appearance which may make detection very difficult. Finally, EM is a very expensive service to provide and requires highly skilled personnel. EM has found a particular niche in the detection of fastidious gastroenteritis viruses such as rota, adeno, astro, Norwalk, and Caliciviruses. It is also used for the rapid diagnosis of herpesvirus infection. It is occasionally used for the diagnosis of human papillomavirus infections and infections by members of the poxvirus family. In addition EM may be used to confirm the results of virus isolation by cell culture such as for parainfluenza viruses.

There are two types of EM methods;- direct or immunoelectron microscopy (IEM). With direct methods, negative staining is normally used which requires little special equipment, in contrast to thin sectioning techniques. The specimens may be used directly or the virus particles may be concentrated before negative staining. Several methods are available for concentration, including differential centrifugation, ammonium persulphate precipitation, and agar diffusion method. Fomvar coated copper grids are used. Immunoelectron microscopy is a means of increasing the sensitivity and specificity of EM and is particularly useful in the following situations;-

1. The number of virus particles present is small.
2. Many different viruses have different morphology e.g. herpesviruses and picornaviruses. IEM may identify the virus
3. In an outbreak situation where the pathogens responsible has been identified so that it may be useful to go back to look at the negative specimens again with IEM.

There are 2 types of IEM, simple IEM, where the specimen is incubated with specific antibody before staining in the hope that the antibody will agglutinate the specimen, and solid phase IEM (SPIEM), where the copy grid is coated with specific antibody which is used to capture virus particles from the specimen.

Electronmicrographs of viruses commonly found in stool specimens from patients suffering from gastroenteritis. From left to right: rotavirus, adenovirus, astroviruses,

3. Complement Fixation Test

The complement fixation test (CFT) was extensively used in syphilis serology after being introduced by Wasserman in 1909. It took a number of decades before the CFT was adapted for routine use in virology. CFT meet the following criteria; it is convenient and rapid to perform, the demand on equipment and reagents is small, and a large variety of test antigens are readily available. However, there is now a trend to replace the CFT with more direct, sensitive and rapid techniques, such as RIAs and EIAs. Although CFT is considered to be a relatively simple test, it is a very exacting procedure because 5 variables are involved. In essence the test consists of two antigen-antibody reactions, one of which is the indicator system. The first reaction, between a known virus antigen and a specific antibody takes place in the presence of a predetermined amount of complement. The complement is removed or "fixed" by the antigen-antibody complex. The second antigen-antibody reaction consists of reacting sheep rbc with haemolysin. When this indicator system is added to the reactants, the sensitized rbcs will only lyse in the presence of free complement. The antigens used for CFT tend to be group antigens rather than type-specific antigens. In order for the CFT to be set up correctly, the optimal concentration of haemolytic serum, complement, and antigen should be determined by titration. The following is a protocol for setting a complement fixation test.

a. Titration of haemolytic serum and complement

Dilutions of complement with 20% difference in concentration are made from 1:30 to 1:279. The following dilutions of haemolytic serum are made: 1:400, 1:800, 1:1600, 1:2000, 1:2400, 1:2800, 1:3200.

The following controls are required:

1. cell control - unsensitized cells only
2. complement control - complement at different concentrations and unsensitized cells
3. haemolytic serum control - sensitized cells only at different concentrations of haemolytic serum

The optimal sensitizing concentration (OSC) of haemolytic serum is the dilution which gives the most lysis with the highest dilution of complement. One haemolytic dose of complement (HD_{50}) is the dilution that gives 50% lysis at the OSC of haemolytic serum. 3 HD_{50} of complement is used for the CFT

b. Titration of antigen and antibody

Antigen at dilutions of 1:2 to 1:512 is titrated against positive serum control. The following controls are incorporated:

1. antigen control - antigen at different concentrations, complement and sensitized cells
2. antibody control - antiserum at different concentrations, complement and sensitized cells
3. cell control well - sensitized cells only
4. complement back titration

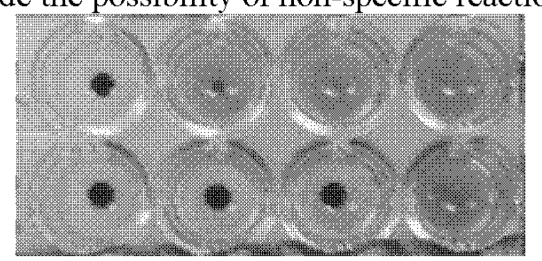
The optimal dose of the antigen is the highest dilution of antigen that gives 75% or more fixation with the highest dilution of antibody.

c. CFT proper

In the CF proper, the haemolytic serum is used at the optimal sensitizing concentration, the complement at $3HD_{50}$, and each individual antigen at the optimal dose. Patients' sera should be inactivated at 56°C for 30 minutes before. Screening is usually carried out at around 1:10 in VBS, if positive, the serum is retested with doubling dilutions of 1:10 to 1:80. Control plates should be prepared with all the antigens used included. The following controls should be present;-

1. Serum control - serum and complement only, to detect any anticomplementary activity in the serum
2. Antigen control - antigen and complement only, to detect any non-specific reaction between antigen and complement.
3. Complement back titration - to check that the complement is used at the correct strength
4. Cell control - sensitized cells only, to check that the cells were suitable for use.

All controls should show complete lysis and in the complement back-titration, the reading should be 0 at the second well and 1 to 2 at the third well. The highest dilution of patient serum that still shows a reading of 3 or 4 is the CF titre. Diagnosis of a recent infection is usually made by the detection of a fourfold or greater increase in titre or by the detection of a high antibody titre from a single specimen (1:80 or above). Where antigens used are grown from yolf sac, the sera should be tested against control yolk sac antigen in order to exclude the possibility of non-specific reaction.



Complement Fixation Test in Microtiter Plate. Rows 1 and 2exhibit complement fixation obtained with acute and convalescent phase serum specimens, respectively. (2-fold serum dilutions were used) The observed 4-fold increase is significant and indicates infection.

Advantages of CFT

1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Cheap

Disadvantages of CFT

1. Not sensitive - cannot be used for immunity screening
2. Time consuming and labor intensive
3. Often non-specific e.g. cross-reactivity between HSV and VZV

4. Haemagglutination Inhibition Test

A wide variety of different viruses possess the ability to agglutinate the erythrocytes of mammalian or avian species. The actual animal species whose erythrocytes could be agglutinated depends on the actual virus. Examples of viruses which could haemagglutinate include influenza, parainfluenza, adenoviruses, rubella, alphaviruses, bunyaviruses, flaviviruses and some strains of picornaviruses. Antibodies against the viral protein responsible for haemagglutination can prevent haemagglutination; this is the basis behind the haemagglutination-inhibition test (HAI). The specificity of the HAI test varies with different viruses. With some viruses such as influenza A, the haemagglutination antigen is the same as the antigen responsible for virus adsorption and thus virus neutralization, and therefore the HAI test is highly specific for the different strains of the virus. With other viruses, the HAI test is less specific eg. flaviviruses, where HAI antibodies against one flavivirus may cross-react with other related flaviviruses. HAI tests are more sensitive than complement-fixation tests but are less sensitive than EIAs and RIAs.

The HAI test is simple to perform and requires inexpensive equipment and reagents. Serial dilutions of patient's sera are allowed to react with a fixed dose of viral haemagglutinin, followed by the addition of agglutinable erythrocytes. In the presence of antibody, the ability of the virus to agglutinate the erythrocytes is inhibited. The HAI test may be complicated by the presence of non-specific inhibitors of viral haemagglutination. and naturally occurring agglutinins of the erthrocytes. Therefore, the sera should be treated before use or false positive or negative results may arise. HAI tests are widely used for the diagnosis of rubella and influenza virus infections. The following is a brief description of the HAI test for rubella.

For rubella HAI testing, one day old chick or goose erythrocytes are used. Bovine albumin veronal buffer (BAVB) is used as the diluent. The HAI test should be carried out using 4 haemagglutination units of rubella antigen. The actual concentration of antigen required should be determined before each HAI test by carrying out a rubella antigen titration from 1:2 to 1:1024. One HA unit is defined as the highest dilution of antigen that gives complete haemagglutination of cells.

In the actual HAI test, the patients' sera are diluted in BAVB from 1:8 to 1:1024. Either V-shaped or U-shaped 96 -well microtitre plate may be used. Non-specific inhibitors of viral haemagglutination may be removed by the treatment of sera before testing by kaolin, RDE, potassium periodate (KIO) or by heat inactivation. Non-specific agglutinins for erythrocytes may be removed by the addition of erythrocytes to the sera prior to testing to allow the erythrocytes to absorb the non-specific agglutinins. This

procedure may be carried out for each serum before testing or may be carried out for sera which had shown agglutination in the serum control wells (serum and erythrocytes only) in a previous HAI test. 4HA of rubella antigen is then added to each well containing diluted test sera except for the serum control wells. A back titration of rubella antigen should be incorporated into the test from 4 HA units to 0.25 HA units. The plate is then allowed to stand at room temperature for 60 minutes after which either 0.5% goose cells or 0.4% chick cells are added to each well and incubated at 4°C for 60 minutes. The plate is then read.

The erythrocytes only control should show a button at the bottom of the well. The serum controls for each serum should show the absence of agglutination. The haemagglutinin back titration should show agglutination at 4, 2 and 1 HA units. A fourfold or greater rise in HAI antibody between acute and convalescent phase sera is indicative of a recent rubella infection.

The advantages of HAI tests are that they are relatively easy and inexpensive to perform. The disadvantages are that HAI tests are not as sensitive as EIAs or RIAs, the actual reading of results is subjective and the reagents should be fresh or else abnormal agglutination patterns may arise which makes the reading and interpretation of the test very difficult. As a result the HAI test for rubella had been replaced by more sensitive and reliable EIA and RIA tests for rubella IgG in many virus diagnostic laboratory

5. ELISA

ELISA was developed in 1970 and became rapidly accepted. A wide variety of assay principles can be used in ELISA techniques. Currently the most important ones are;-

1. Competitive methods
2. Sandwich methods
3. Antibody capture methods

Competitive methods

One component of the immune reaction is insolubilized and the other one labeled with an enzyme. The analyte can then be quantified by its ability to prevent the formation of the complex between the insolublized and the labelled reagent. Advantages of this approach are that only one incubation step is necessary and that the "prozone effect" at high analyte concentrations cannot occur. Disadvantages are that the concentration range in which the analyte can be quantified without sample dilution is rather narrow and that the antigen or antibody (in cases where either may be present in a sample e.g. hepatitis B) produce the same response, and can therefore cannot be distinguished in a one step assay.

Sandwich (Indirect) methods

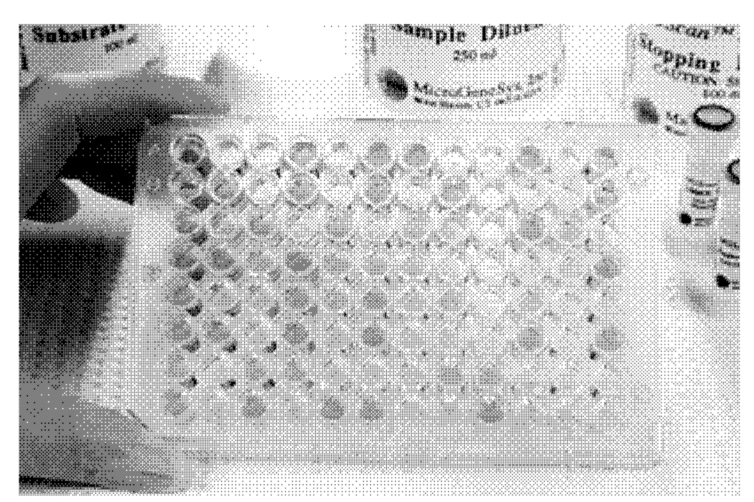
1. The method in which the same component of the immune reaction (e.g. the antibody) is used in the insolubilized and the enzyme labelled form. The other component, the analyte (i.e. the antigen in the sample forms a bridge between the two reagents.)
2. The method in which one component (usually the antigen) is used in an insolubilized form to bind the analyte from the sample (the antibody),which is

subsequently determined by addition of labelled second antibody against the same class of antibody as the analyte antibody or protein A.
In principle, quantification can be achieved over an extremely wide analyte concentration range in such sandwich methods. The "prozone effect" can be avoided in the following ways;- (i) using sequential incubation steps for sample and label, or (ii) by using monoclonal antibodies. Modification of the test in (2) so that antibodies of a specific class such as IgM, can give spurious results if antibodies from other immunoglobulin classes are also present in the sample. Also RF (rheumatoid factor) is known to be a potentially interfering factor.

Sandwich inhibition methods
The sample containing the analyte (usually antibody) is pre-incubated with a fixed amount of its binding partner (ie. the antigen) after which the remaining amount of antigen is determined in a sandwich assay. These methods usually are complicated and have a limited measuring range. The method allows for simultaneous detection of antibody or antigen, if either of these 2 analytes is present in the sample.
Antibody capture methods
These methods used to detect antibodies of specific immunoglobulin subclasses, by first reacting the sample with e.g. insolubilized anti-IgM,and subsequently with either enzyme labelled antigen followed by enzyme linked antibody. Neither antibodies from other immunoglobulin subclasses nor rheumatoid factor interfere significantly in such assays. They are widely used for the diagnosis of acute infections by IgM detection. These assays may be used for detecting IgG and IgA. Considering trends towards simplification of assays and quantification of analytes over a wide concentration range. It must be expected that competitive and sandwich inhibition methods will decrease in importance, and the sandwich and antibody capture methods will be the main assay principles in the future.
Assay Characteristics
The use of monoclonal antibodies has lead to many improvements in ELISA systems.
1. **Higher sensitivity** ;- either by selection of antibodies with a extremely high affinity, or by reduction of the height and variability of the background reaction, which makes very low concentrations of analyte more readily detectable.
2. **Higher specificity** ;- by avoiding the presence of any antibody in the assay system with specific reactivity against non-analyte epitopes, and by selecting combinations of monoclonal antibodies which may further increase specificity.
3. **Higher practicality** ;- e.g. by introducing simultaneous incubation of label, solid phase and sample without risk of "prozone effect".

The enzyme label ;- Most of the assays employ horse-radish peroxidase, alkaline phosphatase, or B-D-galactosidase. The most interesting recent developments has been in new methods to detect these enzymes rather than the use of new enzymes. Fluorimeters were introduced in 1984 for the detection of alkaline phosphatase and B-D-galactosidase. Methods are available to detect horse radish peroxidase by means of chemilumininescence. Fluorimetric and luminometric methods offer higher sensitivity

and a wider measuring range than conventional spectrometry. TMB is gradually replacing mutagenic substrates such as OPD, leading to increased sensitivity and safety.



Microplate ELISA: coloured wells indicate reactivity. The darker the colour, the higher the reactivity

6. Single Radial Haemolysis

Single radial haemolysis (SRH) is routinely used for the detection of rubella-specific IgG. Many sera can be examined simultaneously for evidence of immunity by this technique. SRH has been shown to be both sensitive, specific, and reliable. SRH plates are usually prepared in the laboratory using commercially available reagents, since the short shelf life of the gels makes commercial distribution difficult. Test sera are placed in wells on a plate containing agar with rubella antigen-coated RBC and complement. The presence of rubella-specific IgG is detected by the lysis of rubella antigen-coated RBC. The zone of lysis around the well is dependent on the level of specific antibody present. Besides screening for immunity, SRH can also be used for the diagnosis of acute infection where an increase in the zone size of haemolysis can be demonstrated between acute and convalescent sera. SRH has also been developed for other virus infections such as mumps. However, its main use remains in the screening for immunity against rubella.

SRH plates are prepared as follows. 1% molten agarose is used. Rubella antigen-sensitized sheep rbcs is added to the test plate and unsensitized sheep rbcs to the control plate. Complement is then added to the plates and the molten agarose is allowed to set. The plates are then stored at 4oC until use. Wells are then cut on the test and control plates and serum is added to one well on the test plate and a corresponding well on the control plate. A negative control, a 15 IU/ml control, and a high positive control are used. The plates are then incubated in a moist chamber at 37oC overnight.

The size of the zone of haemolysis around a well containing test serum is compared to that of the 15 IU/ml control. If the zone size is greater than the 15 IU/ml control, then the person is considered to be immune. No zone of non-specific haemolysis should be present on the control plate. Hazy zones of haemolysis may also occur when acute phase sera are tested due to the presence of low avidity antibodies. Some women fail to

produce antibody level of greater than 15 IU/ml even after several vaccinations, therefore many laboratories consider women with a well- documented history of more than one vaccination to be immune, if antibodies are detected by another assay.
Besides SRH, other tests such as ELISA and latex agglutination are also widely used for the screening of rubella antibodies. Sera from patients whose immune status is difficult to determine by SRH may give a clear-cut result with ELISA. Latex agglutination tests have the advantage of speed and simplicity and the technique is also very sensitive. Therefore LA is now used as the screening test for rubella antibodies in many laboratories in preference to SRH.

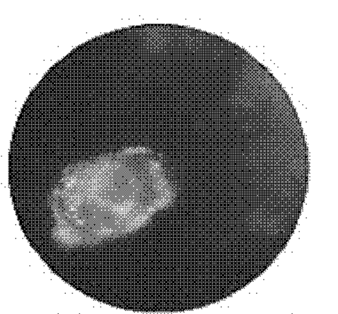
7. Immunofluorescense

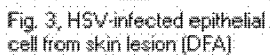
Immunofluorescence (IF) is widely used for the rapid diagnosis of virus infections by the detection of virus antigen in clinical specimens, as well as the detection of virus-specific IgG or IgA or IgM antibody. The technique makes use of a fluorescein- labelled antibody to stain specimens containing specific virus antigens, so that the stained cells fluoresces under UV illumination. In the case of direct IF, the specimen is probed directly with a specific labelled antibody against a particular virus antigen. In the case of indirect IF, the specimen is first probed with a non-labelled specific antibody, followed by a labelled antibody against the first antibody. Direct or indirect IF can be used for the detection of virus antigen, whereas indirect IF is virtually always used for the detection of antibody. Indirect IF possess the advantage of an extra amplification step for the signal, however, it requires an extra step in comparison to direct IF.

Detection of viral antigens

IF is most commonly used for the detection of respiratory viruses in respiratory specimens. Nasopharyngeal aspirates are the best specimens to use and is usually collected from babies less than 12 months old. However, there are no reasons why nasopharyngeal aspirates cannot be collected from older children and adults. A number of respiratory viruses can be detected by direct or indirect IF, including RSV, influenza A and B, adenoviruses and parainfluenza viruses. However, the sensitivities vary greatly between different viruses. The method is most useful in the case of RSV where antiviral treatment is available for severely ill babies. IF is also widely used for the detection of HSV infections, from vesicle lesions and brain lesions, for VZV and CMV infections. However in the case of CMV infection, the sensitivity of IF on clinical specimens directly is low, with the possible exception of the CMV antigenaemia test.
A typical indirect IF procedure for the detection of viral antigens is as follows;- cells from the clinical specimen are immobilized onto individual wells on a slide. Specific polyclonal or monoclonal sera is then added to each well and the slide is incubated at 37oC for 30 to 60 minutes. The slide is then washed 3 times for 5 minutes each with PBS and fluorescein labelled antibody against the first antibody is added. The slide is further incubated at 37oC for 30 to 60 minutes and washed again. The slide is then prepared for microscopy. Specific monoclonal or polyclonal sera raised against the viral antigen can be used. Monoclonal sera offer the advantage of increased sensitivity and specificity. However, one must be certain that it can detect all the different strains of the virus.

IF is highly dependent on the quality of the specimen. In many instances it has proved to be more sensitive than equivalent EIAs. This is because a firm diagnosis can be made on the identification of a few cells only that contain fluorescence of the right colour and with the correct antigen distribution. One of the criticisms of IF is that it is labor intensive and requires highly skilled staff for the reading the specimen.




Fig. 3, HSV-infected epithelial cell from skin lesion (DFA)

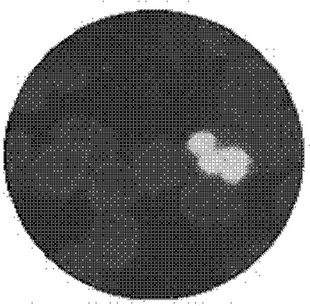
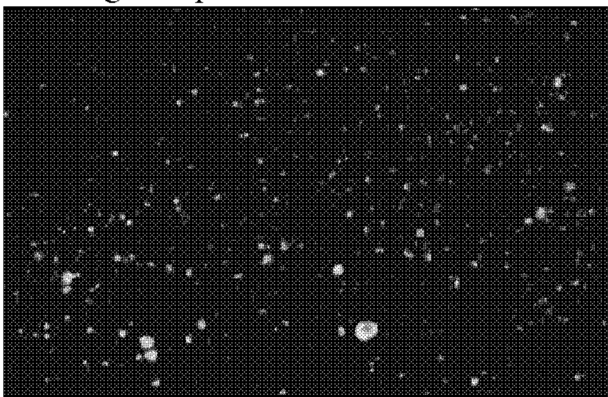


Figure 4 CMV pp65 antigens detected in nuclei of peripheral blood neutrophils



Positive immunofluorescense tests of HSV antigen from epithelial cell and CMV pp65 antigen from peripheral blood neutrophils. (Virology Laboratory, Yale-New Haven Hospital). Right: Positive immunofluorescense test of rabies virus antigen (CDC)

Detection of viral antibodies

IF is probably the simplest serological assay to set up. It simply requires virally infected cells that express viral antigens and a fluorescein-labelled antiserum against human immunoglobulin. IF can be used to detect IgG, IgM and IgA. Being very easy to set up, it is often the first and only serological assay available for newly discovered viruses, in particular arboviruses. IF is extensively used for the diagnosis of EBV infections and is also routinely used for other viruses such as VZV.

8. Neutralization

Neutralization of a virus is defined as the loss of infectivity through reaction of the virus with specific antibody. Virus and serum are mixed under appropriate condition and then inoculated into cell culture, eggs or animals. The presence of unneutralized virus may be detected by reactions such as CPE, haemadsorption/haemagglutination, plaque formation, disease in animals. The loss of infectivity is bought about by interference by the bound Ab with any one o the steps leading to the release of the viral genome into the host cells. There are two types of neutralization;-

Reversible neutralization - The neutralization process can be reversed by diluting the Ab-Ag mixture within a short time of the formation of the Ag-Ab complexes (30 mins). It is thought that reversible neutralization is due to the interference with attachment of virions to the cellular receptors eg. the attachment of the HA protein of influenza viruses to sialic acid. The process requires the saturation of the surface of the virus with Abs.

Stable neutralization - with time, Ag-Ab complexes usually become more stable (several hours) and the process cannot be reversed by dilution. Neither the virions or the

Abs are permanently changed in stable neutralization, for the unchanged components can be recovered. The neutralized virus can be reactivated by proteolytic cleavage.

Stable neutralization has a different mechanism to that of reversible neutralization. It had been shown that neutralized virus can attach and that already attached virions can be neutralized. The number of Ab molecules required for stable neutralization is considerably smaller than that of reversible neutralization, Kinetic evidence shows that even a single Ab molecule can neutralize a virion. Such neutralization is generally produced by Ab molecules that establish contact with 2 antigenic sites on different monomers of a virion, greatly increasing the stability of the complexes. An example of stable neutralization is the neutralization of polioviruses, whereby, the attachment of the antibody to the viral capsid stabilizes the capsid and inhibits the uncoating and release of viral nucleic acid.

Viral evolution must tend to select for mutations that change the antigenic determinants involved in neutralization. In contrast, other antigenic sites would tend to remain unchanged because mutations affecting them would not be selected for and could even be detrimental. A virus would thus evolve from an original type to a variety of types, different in neutralization (and sometimes in HI) tests, but retaining some of the original mosaic of antigenic determinants recognizable by CFTs. Because of its high immunological specificity, the neutralization test is often the standard against which the specificity of the other serological techniques is evaluated.

Before the neutralization test is carried out, the known components that are to be used must be standardized. To identify a virus isolate, a known pretitred antiserum is used. Conversely, to measure the antibody response of an individual to a virus, a known pretitred virus is used. To titrate a known virus, serial tenfold dilutions of the isolate is prepared and inoculated into a susceptible host system such as cell culture or animal. The virus endpoint titre is the reciprocal of the highest dilution of virus that infects 50% of the host system eg. 50% of cell cultures develop CPE, or 50% of animals develop disease. This endpoint dilution contains one 50% tissue culture infecting dose ($TCID_{50}$) or one 50% lethal dose (LD_{50}) of virus per unit volume. The concentration of virus generally used in the neutralization test is 100 $TCID_{50}$ or 100 LD_{50} per unit volume.

The antiserum is titrated in the neutralization test against its homologus virus. Serial twofold dilutions of serum is prepared and mixed with an equal volume containing 100$TCID_{50}$ of virus. The virus and serum mixtures are incubated for 1 hour at 37oC. The time and temperature for incubation varies with different viruses. The mixtures are then inoculated into a susceptible host system. The endpoint titration contains one antibody unit and is the reciprocal of the highest dilution of the antiserum protecting against the virus. Generally 20 antibody units of antiserum is used in the neutralization tests.

9. IgG Avidity Tests

Rubella reinfection can occur, especially in those whose immunity were induced by vaccination rather than by natural infection. However, reinfection by rubella during the first trimester of pregnancy is thought to pose minimal risks to the fetus. Cases of CRS arising from rubella reinfection have rarely been reported and termination of pregnancy

is not recommended. Therefore, it is important to distinguish reinfection from primary infection by rubella during the first trimester of pregnancy. In the absence of reliable confirmatory tests, needless abortions may result.
Rubella specific IgM can be detected following both primary and reinfection. Although in the latter case, it is likely to be more transient and of a lower level One solution for the differentiation of primary from reinfection could be the measurement of the antigen-binding avidity of specific IgG. The avidity of IgG is low after primary antigenic challenge but matures slowly within weeks and months.

Methods

1. **Semi-quantitative test**; - This is based on the recognition of a characteristic pattern in the radial haemolysis test. The zone of haemolysis was assessed; Haemolytic zones with "soft" diffuse outer margins are produced by antibodies of low avidity. Haemolytic zones with a discrete outer margins (designated "ordinary") are produced by antibodies of high avidity. Zones that were neither diffuse nor discrete are classified as equivocal.
2. **Quantitative test "Avidity ELISA"** ;-

Elution principle - in this test a mild protein denaturing agent such as urea or diethylamine (DEA), is added to the antibody - antigen mixture. Antibodies of low avidity are more likely to dissociate from the antigen-antibody complexes than those of higher avidity. The rest of the test is as for a normal ELISA. The results obtained in the presence and absence of the denaturing agent is compared and a ratio is derived. Low avidity antibodies will have a much higher ratio than high avidity antibodies.

a. The sera to be tested is diluted at various concentrations in the presence and absence of DEA and assayed for IgG1 and IgG3. The optical densities obtained were plotted. The highest OD (V) was noted and halved (V/2) and the distance between the OD curves at V/2 was measured as the DEA shift value.

Hedman et al (1989) measured the IgG avidity from 64 sera. According to their avidity IgG ELISA, 29 had low avidity Ab, 29 had high avidity Ab and 6 were borderline. Comparison with known clinical records showed that all patients with low avidity Ab had recent primary infection. Those with high avidity had previously been immune. Amongst this group were 4 that had originated from confirmed reinfections. Of the equivocal sera, 5 out of 6 were obtained within 2 months of primary infection.
Thomas and Morgan-Capner (1988) measured the avidities of Rubella specific IgG1 and IgG3. IgG3 is rarely demonstrated in reinfection. They tested sera from 24 patients who were immunized or infected in the distant past, 66 who recent rubella primary infection, 11 from those with symptomatic reinfection and 64 from those with asymptomatic reinfection. For IgG1 the DEA shift value was< 0.6 for cases of rubella in the distant past, compared with 0.8 for the first month after primary infection. The maximum DEA shift value for the sera from cases of reinfection was 0.65. No serum from cases of rubella in the distant past contained sufficient specific IgG3 to estimate avidity. The sera collected within one month of the onset of rubella gave DEA shift values of 0.7 compared to sera from reinfection. In general, the elution-principle test is

more sensitive for past infection but less sensitive for recent infection. Whereas the dilution-principle test is more sensitive for recent infection.

10. Molecular Techniques

Molecular biology techniques for the direct detection of viral genomes in the specimen will play an increasingly important role in the clinical virology laboratory in the 21st century. Molecular techniques can be divided into two categories: those that do not involve amplification i.e. hybridization with nucleic acid probes, and those that involve amplification e.g. PCR, LCR, NASBA etc.

Nucleic Acid Probes

Nucleic acid probes are segments of DNA or RNA that have been labeled with enzymes, antigenic substrates, chemiluminescent moeities, or radioisotopes. They can bind with high specificity to complementary sequences of nucleic acid. Probes can be directed to either DNA or RNA targets and can be from 20 to thousands of bases long. The presence and the quantity of hybrids after hybridization is determined by the detection of the label. Probes are usually synthesized by one of the following three methods.

1. Oligonucleotide probes - these are usually less than 50 bases long and are synthesized chemically
2. PCR - will produce probes from 50 to several hundred bases long
3. Cloning - will produce probes several thousand bases long e.g. probe for complete HBV genome

Altering the stringency of the reaction will alter the sensitivity and specificity of the hybridization. Stringency relates to the number of mismatched base pairs that can be tolerated when two nucleic acid molecules come together to form a double stranded molecule. Stringency is affected by several variables, including the temperature, salt concentration, and the pH of the hybridization reaction. High stringency is achieved by using buffers of low salt concentration or by conducting the hybridization reaction and stringency washes at higher temperatures. The higher the stringency of reaction, the less likely it is for mismatched base pairs to stay together.

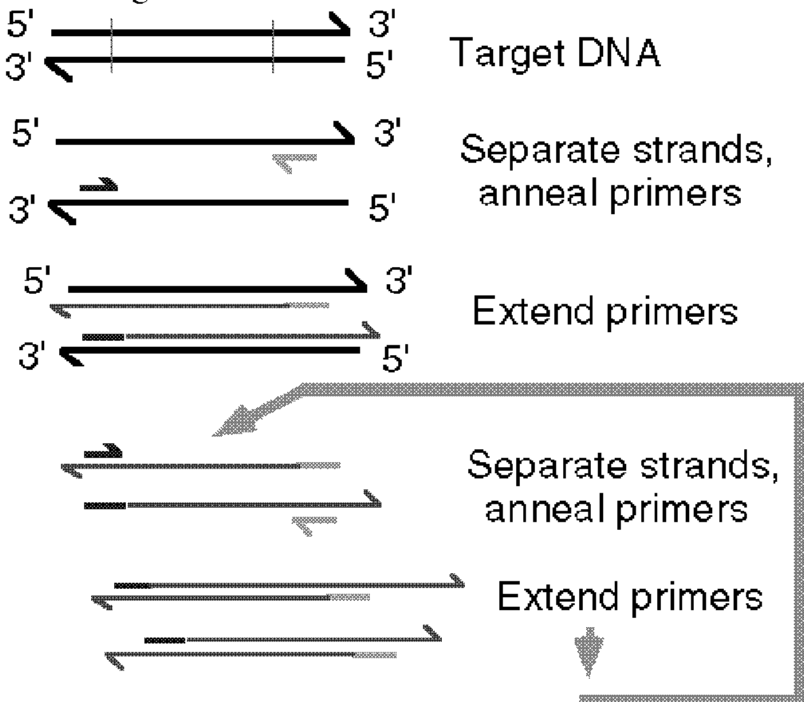
The hybridization reaction may be carried out completely in solution phase whereby both the target nucleic acid and the probe are free to interact in the reaction mixture. Solution hybridization has the advantages of being rapid to the carry and carry a higher sensitivity than solid phase hybridization. This is the approach taken by Abbott with their quantitative HVB-DNA assay. Nucleic acid bound to a solid surface are still available to participate in hybridization reactions. However, the sensitivity tends to be lower than that of liquid hybridization. However, this technique greatly facilitates the handling of multiple samples. The dot-blot and sandwich hybridization assays are commonly used in this respect. In situ hybridization assays, in which whole cells or tissue sections are put through the hybridization process has become an important research tool.

Despite having been around for many years, hybridization assays are still not in common use in the clinical virology laboratory. The main reason is that its sensitivity is not usually higher than far simpler conventional virological techniques such as cell

culture and viral antigen detection.

Polymerase Chain Reaction

PCR allows the in vitro amplification of specific target DNA sequences by a factor of 10^6 and is thus an extremely sensitive technique. It is based on an enzymatic reaction involving the use of synthetic oligonucleotides flanking the target nucleic sequence of interest. These oligonucleotides act as primers for the thermostable Taq polymerase. Repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (50°C), and primer extension (70°C) result in the exponential production of the specific target fragment. Further sensitivity and specificity may be obtained by the nested PCR technique, whereby the DNA is amplified in two steps. In the first step, an initial pair of primers is used to generate a long sequence that contain the target DNA sequence. A small amount of this product is used in a second round of amplification, which employs primers to the final target DNA.



Schematic of Polymerase Chain Reaction

Detection of DNA sequence product of the PCR assay may be performed in several ways. The least sensitive and specific method is to size fractionate the reaction product on an agarose or acrylamide gel and stain the DNA with ethidium bromide. A more sensitive technique involves the attachment of DNA to a membrane through dot or slot-blot techniques followed by hybridization with a labelled homologous oligonucleotide
probe. Alternatively, the PCR product may be probed directly by liquid oligom eric hybridization. However, these techniques provides no information on size of

the amplified product and thus could not exclude the possibility that the product originated from a region of the human genome which exhibits homology with the target CMV sequence. The most sensitive and specific detection methods result from combining the size information of gel electrophoresis with the improved sensitivity and specificity of hybridization techniques. This may be achieved by gel electrophoresis followed by Southern transfer and hybridization, or through liquid oligomeric hybridization followed by gel electrophoresis.

Advantages of PCR:

1. Extremely high sensitivity, may detect down to one viral genome per sample volume
2. Easy to set up
3. Fast turnaround time

Disadvantages of PCR

1. Extremely liable to contamination
2. High degree of operator skill required
3. Not easy to quantitate results
4. A positive result may be difficult to interpret, especially with latent viruses such as CMV, where any seropositive person will have virus present in their blood irrespective whether they have disease or not.

The first three problems are being addressed by the arrival of commercial closed systems such as the Roche Cobas Amplicor which requires minimum handling. The use of synthetic internal competitive targets in these commercial assays has facilitated the quantification of results. Otherwise, the same problems remain with in-house PCR assays. The problem with contamination is particularly acute with nested PCR assays and they should be avoided in the clinical setting as far as possible. The sensitivity would normally be sufficient if one use a single PCR reaction followed by hybridization with a specific oligonucleotide probe. This is the approach taken by commercial assays. The fourth problem is more difficult to resolve but it is generally found that patients with active CMV disease has a much higher viral load than those who do not. Therefore, it is simply a case of finding the appropriate cut-off.

Other Amplification Techniques

Following the heels of PCR, a number of alternative in-vitro amplification techniques have been developed, of which some are now available commercially. Examples of these alternative techniques include ligase chain reaction (LCR), nucleic acid sequence based amplification/isothermal amplification (NASBA), strand displacement amplification, Q-PCR Replicase method, and branched DNA probes. Of these techniques, LCR, NASBA and branched DNA are now available commercially in an automated or semi-automated format. A NASBA assay is available for the quantification of HIV-RNA (Organon), and an LCR assay is available for the detection of chlamydia (Abbott). Branched DNA assays are available for the detection of quantification of HIV-RNA, HBV-DNA, and HCV-RNA (Chiron).

With the exception of the branched DNA probe, all these techniques involve exponential amplification of either the target nuclei acid or the probe. Therefore, they

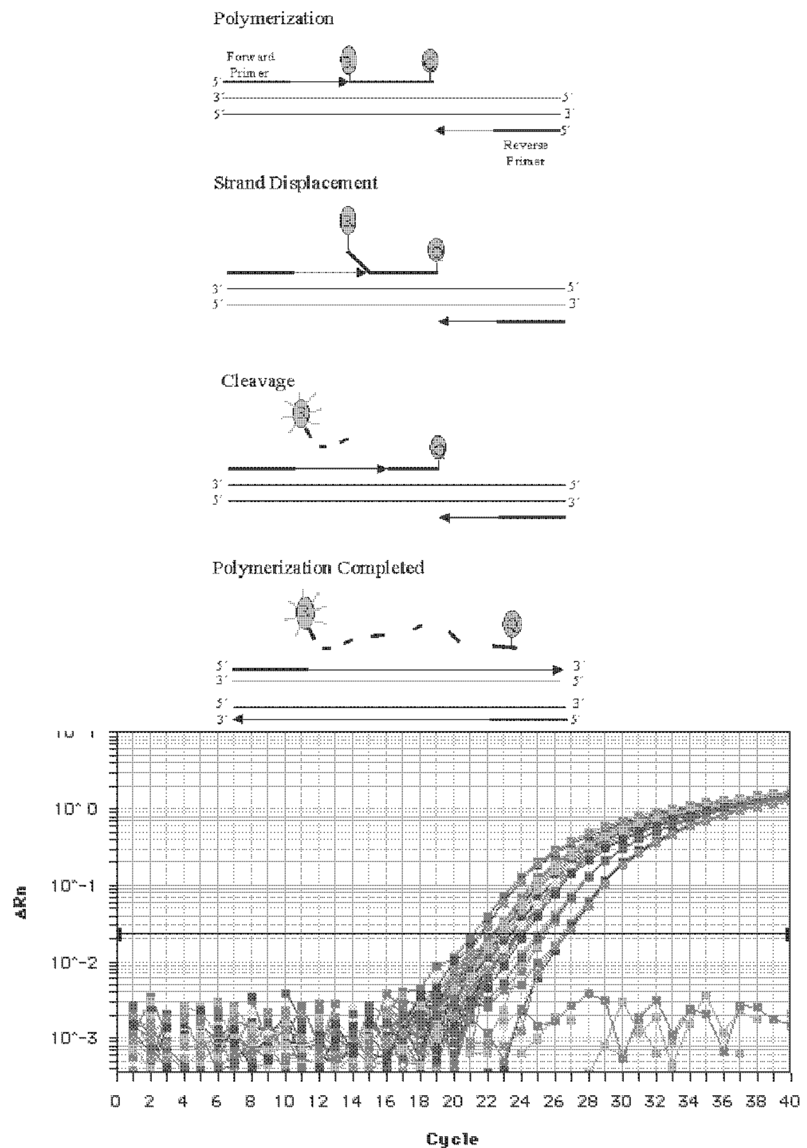
are all as susceptible to contamination as PCR. The branched DNA system is really an intermediate between classical hybridization techniques and the newer in-vitro amplification techniques. It is not as sensitive as those techniques which involve exponential amplification but is considerably more sensitive than the classical hybridization techniques. Below is a brief summary of the features of the different amplification methods available.

Quantitative PCR

(Q-PCR) is used to directly measure abundance of known mRNA in
minute samples. TaqMan is described but there are other variations (SyBr Green).
Works
with small amount starting material. Expensive machine (facilities)

1. Two fluorescent dyes, a reporter (R) and a quencher (Q), are attached to the TaqMan probes. The 3' end of the probe is blocked, so it is not extended during the PCR reaction.
2. When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the probe anneals to the interior of the PCR product
3. During polymerization from the upstream primer Taq DNA polymerase cleaves the reporter dye from the probe via its 5'-3' exonuclease.
4. Separated reporter dye emits its fluorescence which is measured by the Q-PCR machine.
5. The amount of fluorescence measured is proportional to the amount of PCR product made.
6. Score cycle number at where signal equals threshold value
7. threshold value is generally set at 10 to 20-fold average noise, during geometric amplification (linear portion of log plot)
8. since each RNA species amplifies at a slightly different efficiency a standard curve is needed to calculate absolute amounts of RNA. Relative amounts of RNA between samples can be calculated from 6.

Amplification Procedures with labeled nucleotides



Quantitation Diagram compared with controls

DNA microarrays

A. DNA microarrays (DNA chips) allow for the simultaneous screening for changes in expression of thousands of genes .

B. DNA from thousands of genes is arrayed on small chip (glass etc.)

1. PCR products (cDNAs, promoters, or other specialized DNAs)

2. Photolithography (9-21). Build up oligos using light activated precursors and masking.

C. Probes: cDNA made by RT and fluorescent labeled nucleotides (generally two populations)

D. Changes in fluorescent color that indicates changes on gene expression. Examples: developmental, diseased vs. normal.

Laboratory Strategy in diagnosis of Infections

These are detailed outlines laboratory strategy in dealing with the following types of sample or infective syndrome:

1. Blood culture
2. Upper respiratory tract infections, including throat. nose, ear, eye infections
3. Lower respiratory tract infections
4. Wound, skin and deep sepsis
5. Genital tract infections
6. Meningitis
7. Gastrointestinal infections
8. Urinary tract infections
9. Pyrexia of unknown origin.

1. BLOOD CULTURE

Culture of patient's blood is one of the most important investigations in clinical microbiology.

Uses

Blood culture is requested mainly in two clinical situations:

(1) where the possibility of septicemia or bacteriaemia is suggested by the presence of fever, shock or other symptoms occurring in association with a known or suspected local infection such as sepsis in a surgical wound, puerperal sepsis, pneumonia, meningitis, osteomyelitis or endocarditis.

(2) where it is one of the procedures required in the investigation of a fever difficult to diagnose because of the absence of signs of a specific infection or local infective lesion, i.e. a pyrexia of unknown origin (PUO).

Collection of sample.

To minimize difficulty in interpretation, every care must be taken to prevent contamination of the specimen during its collection from the patient and its in the laboratory. They should be instructed how to disinfect the skin over the vein, to use a fresh sterile syringe for the venepuncture and to replace the needle used for the venpuncture with a fresh sterile needle before inoculating the blood into the culture bottles They should hold the needle by its butt, not shaft, either with sterile forceps or with the fingers covered with a dry sterile rubber glove, and should take care to avoid contaminating themselves or the outside of the culture bottles with potentially infective blood.

They should also be warned that contamination is very likely. if the sample is collected from an indwelling peripheral venous catheter or a long central venous line instead of from a fresh venepuncture. In some cases such lines themselves become colonized with skin commensal bacteria which are then seeded into the circulation.

Culture Method

It is necessary to culture at least several milliliters of blood diluted I in 5 to 1 in 10 in a culture medium.

This is conveniently done b) inoculating 5-10 ml blood into 50 ml liquid medium in a bottle of 90-125ml capacity. To maximize the chances of detecting scanty bacteria, a

larger volume, e.g. 10-20 ml, of blood should be collected and half of it should be inoculated into each of a 'set' of two such culture bottles. The use of two bottles has several further advantages: they may contain different media or be incubated different atmospheres and scanty contaminants are likely to grow in only one of them.

The bottles of sterile medium should be tightly closed with a perforated aluminum or plastic cap over an entire rubber washer, and these should be covered and kept sterile till the moment of inoculation by a metal foil cover. After removal of the foil, the blood is inoculated into the bottle by inserting the syringe needle through the rubber washer, the bottle remaining unopened and unexposed to the entry of airborne dust. Instructions for the collection of the sample and its introduction into the bottle should be given on a printed label attached to the bottle.

Types of Culture media.

The chance of detecting scanty or exacting bacteria will be maximized if a variety of rich media are seeded from each sample and different media are used for aerobes and anaerobes. But use of a single, good all-purpose medium will give nearly as many positive results, saves in labor and cost of materials, and reduces the risk of confusion in the issue and use of the culture bottles.

Traditional Bottles

-If a single, all-purpose medium is to be used, it should be richly nutritive and capable of supporting the growth of both strict aerobes and strict anaerobes when incubated sealed and unexposed to an external atmosphere. A good choice is a Robertson-type cooked-meat medium based on brain heart infusion broth and having at its foot a layer of pieces of lean meat about 3 cm deep . The inoculated blood adds nutrients, including X and V factors, and small amounts of O2 and CO2. When the bottle is incubated while still sealed, most anaerobes will find suitable conditions for growth among the pieces of meat and strict aerobes will grow at the surface of the broth where they have access to oxygen in the overlying air. If the bottle has been autoclaved with its cap tightly closed, its original content of air will still be present. If it has been autoclaved with its cap loose, the air expelled during autoclaving will have been replaced by air drawn into during the subsequent cooling, unless the washer sucked tightly on to its mouth.

If two bottles have been seeded from the sample, their caps may be loosened and one of them incubated in air plus 5-10% CO2, the other in an anaerobic jar, but the advantages of this procedure over that of incubating both bottles still sealed have not been proven.

When the presence of certain exacting organisms is indicated, specially suitable media and methods of incubation should be used Sabouraud broth incubated at 28°C for up to 10 days for yeasts and fungi.

Recent Blood Culture Bottles

-Casteneda-rvpe bottles. The difficulties of making many repeated examinations of blood cultures may be avoided by the use of bottles of the Casteneda type, which contain an agar surface above the level of the culture fluid. Repeated attempts at subculture may be made with a minimum of labor and without ever opening the bottle and exposing it to the risk of contamination.

The bottle is momentarily tilted to run some culture fluid over the agar surface and then re-incubated until colonies are seen to appear on the latter.
-A commercially available kit (Roche) supplies the agar medium in a clear plastic container that is attachable to the mouth of a conventional blood culture bottle on its receipt in the laboratory.
-Another kit (GIbco) contains the agar slope in a parallel chamber of a double bottle into which some of the seeded broth can be tipped.
-Gas capture system. A simple blood culture system recently developed by Oxoid obviates the need for expensive automated equipment, demonstrates both aerobic and anaerobic organisms in a single culture bottle, and permits frequent, easy observation of the onset of growth. The blood culture bottle contains a medium specially formulated to promote gas production on the growth of any aerobic or anaerobic organism and the gas pressure forces medium into a reservoir where it can be observed visually in the course of incubation.

Automated systems

These systems employ equipment that automatically detects an early sign of bacterial growth in a special blood culture bottle. The system most used is Bactec (Becton Dickinson).

It depends on the release of carbon dioxide (C02) into the atmosphere in the culture bottle by the bacterial degradation nutrients in a special culture medium and PH of the bottle becomes acidic leading to release of fluorescent signal detected by specific sensor in Bactec system.

Separate bottles of special medium are supplied for aerobic and anaerobic culture, and bottles of medium containing resins to inactivate any antibiotics that may be present in the specimen.

Normally 10 ml blood would be collected in a syringe and up to 5 ml injected through their rubber caps into aerobic and anaerobic culture. bottles containing 30 ml medium. Up to 60 bottles of the one kind seeded from different patients are loaded into an instrument in which they are incubated, agitated and periodically flushed with an appropriate gas (air + CO2, or N2 + H2 + C02) through two heat-sterilized needles inserted automatically through the rubber cap). It can indicate early bacterial growth, which is often detectable on the same day as the specimen is received in the laboratory.

When growth is thus first detected, the bottle is removed and examined in the usual way by filming and subculture. Negatively reacting bottles do not need to be filmed or subcultured at any stage.

Examination of blood cultures.

-Depending on the number of bacteria in the bloodstream and other factors, the first appearance of growth may take place after as little as a few hours or only after a longer period of 1 or a few days or occasionally 1 or weeks and medium incubated at 37°C for up to 6 weeks for Brucella.

As it is desired to detect any positive culture as early as possible and yet not miss any late-growing culture the ideal procedure is to make repeated examinations of the culture bottle at different timer during its incubation from 4 h to 2 weeks longer. if. however,

many requests for blood culture are received each day, the amount of labor required for making more than a few examinations on each bottle may be excessive . Moreover, every opening of a bottle for examination affords an extra opportunity for contamination, and overlapping examinations on successive batches of bottles on the same day increase the chance of confusion and mistakes.

- Trials of different schedules suggest that a minimal, reasonably effective procedure is the making of just two examinations on each bottle, the first after incubation at 37°C overnight (i.e. for 12-24 h), the second after 4-7 days. Only very few cultures negative at 4 days become positive on longer incubation. When a report is required urgently on a suspected septicemia, an additional examination may be made after only 4 h incubation.

Positive findings at that stage are available for earlier reporting to the clinician and are less likely to be due to the growth of scanty contaminants than those obtained after longer incubation. The negative cultures, which constitute the majority of all those received, have to be examined again at 18-24 h and 4-7 days. requiring a total of three examinations. The extra work of the early. First-day examination may be minimized by confining it to the making of subcultures and omitting the more time-consuming examination by Gram film.

It is recommended that on each subsequent occasion of examination, the blood culture should be examined both by a Gram film and by subculturing on two plates of blood agar, the one incubated in air plus 5-10% C02, the other anaerobically in nitrogen/hydrogen plus 5-10% C02. If the presence of Meningococcus or Haemophilus seems likely, e.g. in suspected septicemia meningitis, a heated-blood (chocolate) agar plate may be substituted for the aerobic plain blood agar plate. Gram filming is particularly valuable when the first examination is made only after overnight incubation, i.e. after 12-24 h, for when bacteria are seen to be present in the film, a preliminary report may be phoned to the physician at once, without the need to wait another day to see growth of the bacteria in the subcultures.

The procedures of filming and subculture must be performed in such a way as to minimize the opportunities for contamination of the blood culture with bacteria from the air, fingers or equipment. The bacteriologist should gently mix the contents of the bottle without wetting the inside of the cap, then remove the cap, withdraw 0.5-1ml of the culture fluid into a sterile Pasteur pipette, and at once replace the cap. (Contamination of the bottle is less likely to occur during one dip with a pipette than during three with an inoculating loop.) He should then let a large drop of the fluid fall from the pipette on to each subculture plate and finally spread a drop on a slide for Gram staining. Lastly, he should spread the drops on the plates over large "well" areas and streak them out on the remainder.

Gram film. The Gram film should be examined as soon as the subcultures have been set up, and positive findings reported at once to the physician so that the morphological type of the organism present may serve as a guide to his initial choice of antibiotic. The finding will also indicate to the bacteriologist what further subcultures and tests should be seeded from the positive bottle. Two extra plates should be seeded confluent and the appropriate antibiotic disks applied to them for sensitivity testing on aerobic and

anaerobic incubation. Optochin and bacitracin disks may be used on the subculture plates to identify organisms resembling Pneumococci and Streptococci. A MacConkey plate may be seeded to assist in the identification of Gram-negative bacilli, a DNase test plate for staphylococci, an aesculin-bile plate for diphtheroid bacilli (to exclude Listeria, which is positive), and a Nagier plate for bacilli resem Wing Clostridium perfringens.
A possible cause of error must be borne in mind when reading the Gram film. Some media, particularly some batches of meat pieces incorporated in cooked meat medium, may be contaminated with saprophytic bacteria, which, although they have been killed in the sterilization procedure , may be sufficiently numerous to be seen in the film, if only one morphological type is seen, its presence may lead to the making of a false provisional report, but if a mixture of types is seen, their origin as contaminants will be suspected. The error will be recognized next day when the subcultures will be found to be negative.
Examination of subcultures. The plates should be inspected after incubation for 18-24 h, when any growth should be examined in a Gram film and by the setting up of appropriate identifying and sensitivity tests. Plates showing what is possibly very slight growth and plates seeded from film-positive bottles but still devoid of growth should be re-incubated for up to three more days before being discarded as negative.
Reports. Relevant positive findings should be reported by phone to the physician as soon as is practicable and, if requested. advice should be given on the choice of antibiotic therapy. A written report should be issued when the examinations made after the first 12-24 h of incubation have been completed. For positive cultures, the species and drug sensitivities should be reported, and for negative cultures, the absence of growth and a note that a further report will be issued if growth appears on longer incubation.
When Staphylococcus albus or a commensal type of Diphtheroid bacillus is found and no predisposition to opportunistic infection is known, the report should state that the organism is probably a contaminant from the patient's skin and omit the drug sensitivity findings. If, however, the patient has suspected endocarditis, an artificial heart valve, an intravenous catheter or a cerebrospinal fluid shunt, or has had heart surgery, or is immunosuppressed or on cytotoxic drugs, the finding of the organism and its sensitivities should be reported, but with the caution that it may possibly be a contaminant from the patient's skin.

The isolation of a bacterium from the blood of a patient with a local infection such as wound sepsis or pneumonia is valuable firstly in indicating the urgent need for antibacterial therapy, secondly in revealing the species of bacterium against which therapy should be directed. and thirdly in providing a culture for the performance of in-vitro drug sensitivity tests. Culture of a specimen from a local site of infection, e.g. a wound swab or sputum specimen, often yields a mixture of contaminating commensal bacteria and potential pathogens, the clinical significance of which is unclear, but the demonstration of one of these species in the blood indicates that it at least is an invader of clinical significance. If, for instance, a Pneumococcus is cultured from sputum from a patient with suspected pneumonia, it may have been derived from its site of commensal

carriage in the throat and not from the lungs, which may instead be infected with an undetected virus or Mycoplasma. But if the Pneumococcus is cultured from the blood, it may be concluded that it is probably present as a pathogen in the lungs and that the patient certainly requires anti-pneumococcal therapy.

Contamination:

A difficulty in interpreting results arises from the liability of the specimen to be contaminated with organisms that are commensals or common contaminants on the skin. Staphylococci albus are the commonest contaminants, but Dphtberoid bacilli, Coliform bacilli, Anthracoid bacilli, Clostridium perfringens, candida and other organisms are sometimes present. and the finding of one of these organisms in a blood culture should be viewed with the strong suspicion that it is a contaminant from the skin. But in a patient with debility, immune deficiency, an intravascular line, a prosthetic implant or some other condition favoring an opportunistic infection, the saprophytic or commensal organism isolated may indeed have an important pathogenic role. Consideration must be given to the probability of its having such an opportunistic role in the circumstances of each particular case. To this end, culture should be attempted on several separately collected samples of blood, for the isolation of the same species of organism from repeated specimens suggests that it is probably present as a cause of infection and not merely as a contaminant. This probability is strengthened if it can be shown that the different isolates are identical with one another In their pattern of antibiotic sensitivities, biotype or phage-type.

Examination in the laboratory should be planned with a view to minimizing the opportunities for contamination of the specimen or its subcultures with airborne dust or from the bacteriologist's skin or equipment. However it should be noted that despite insistence on aseptic precautions, most laboratories report finding contamination in 1—5% of the blood cultures they examine.

False-negative results. The media and methods used for blood culture should be such as to support growth of nutritionally exacting organisms, and both strict aerobes and strict anaerobes as well as facultative organisms. Even SO, a single negative culture should not be taken as proof that a patient does not have a blood infection. in bacteriaemic illnesses the number of bacteria in the blood may vary rapidly between many and few or none as they are introduced intermittently from a local infective focus and removed by the reticuloendothelial tissues. To maximize the chances of isolation when the bacteria may be scanty, a large volume of blood, e.g. 10—20 ml, from each collection should be cultured and a number of samples should be collected on separate occasions. Thus, in attempting to discover the organism causing a suspected endocarditis, at least six samples collected at intervals of several hours in the course of 3-6 days should be cultured.

Antibiotics in the blood: The blood sample should be diluted between 1 in 5 and 1 in 10 in the culture medium in order to reduce the concentration of natural antibacterial constituents to a sub-effective level. The dilution also reduces the concentration of any therapeutically administered antibiotic. But when an antibiotic is present in the

undiluted blood at more than 10 times its minimum inhibitory concentration, t may still, after dilution, prevent the growth of viable bacteria in the specimen.

For this reason, every attempt should be made to ensure that samples of blood are collected before the start of antibiotic therapy. If an antibiotic has already been given to the patient, the collection of the blood sample should preferably be delayed until at least 24 h after a dose has last been given. Otherwise, it may be possible to add an antibiotic-inactivating agent to the culture bottle. Para-aminobenzoic acid used to be added routinely to blood culture media to neutralize any sulphonamide administered. If the patient has been treated with a β-Iactam antibiotic (a penicillin or cephalosporin), a broad-spectrum β-lactamase should be added to the culture bottle as soon as possible after introduction of the blood, e.g. 0.2ml of Wellcome penicillinase to a bottle containing 50 ml medium. Special blood culture media are commercially available that contain resins that are said to be capable of inactivating a variety of antibiotics (Marion).

2. UPPER RESPIRATORY INFECTIONS

The commonest respiratory infections are localized in the oropharynx, nasopharynx and nasal cavity, causing sore throat, nasal discharge and often fever, but the throat pathogens may also spread to infect the larynx, causing hoarseness, the middle ear, causing otitis media with earache, a paranasal sinus, causing sinusitis with pain in the face or head, and the eye, causing conjunctivitis or keratitis. The upper respiratory tract may also be involved in wider respiratory or generalized infections such as whooping cough, influenza, measles and infectious mononucleosis.

In most cases the primary infection is viral, though the causal virus is generally not demonstrated, and there is often concomitant carriage or secondary infection with one of the potential bacterial pathogens commonly present in the nasopharynx, e.g. Pneumococcus, Haemophilus infiuenzae (H. infiuenzae). Staphylococcus aureus and Streptococcus pyogenes (S.pyogenes). Drug-resistant Coliform bacilli or yeasts may come to dominate the throat flora in patients receiving antibiotics, but their presence is generally of little pathological significance.

Streptococcal pharyngitis

The only common primary bacterial cause of sore throat is S. pyogenes, which is found in about 30% of cases of pharyngitis, with or without tonsillitis. Its detection is the main purpose of the bacteriological examination of throat swabs, for it is the only common throat pathogen for which antibiotic therapy is clearly indicated. When it is thought or shown to be present, benzyipenicillin and procaine penicillin should be given by intramuscular injection and followed by phenoxymethyl penicillin given orally for 7-10 days; commonly, the injections are omitted. Erythromycin should be given to patients allergic to the penicillins. Effective therapy should cause rapid amelioration of symptoms, e.g. within 24-48 h, and prevent serious complications such as otitis media and rheumatic fever. Streptococcal pharyngitis cannot be distinguished clinically from viral pharyngitis, so whenever practicable throat swab examinations should be made on patients with sore throat.

In only a few cases are there clinical indications requiring the examination of throat swabs for other pathogens, such as the diphtheria bacillus , Vincent's organisms. Candida or Gonococcus and in the absence of such indications swabs from pharyngitis should be examined only for S.pyogenes. If the presence of commensal nasopharyngeal residents like H. infiuenzae. Pneumococcus, S. aureus and coli-form bacilli is reported to the physician, he may be induced to give inappropriate antibiotic therapy.

Method of Throat swabs. A plain, albumen-coated or charcoal-coated cotton-wool swab should be used to collect as much exudates as possible from the tonsils. posterior pharyngeal wall and any other area that is inflamed or bears exudates. If the patient permits, the swab should be rubbed with rotation over one tonsillar area, then the arch of the soft palate and uvula, the other tonsillar area, and finally the posterior pharyngeal wall. An adequate view of the throat should be ensured by good lighting and the use of a disposable wooden spatula to pull outwards and so depress the tongue. The swab should be replaced in its tube with care not to soil the rim. If it cannot be delivered to

the laboratory within about 1 h, it should be placed in a refrigerator at 4°C until delivery or, preferably, it should be submitted in a tube of transport medium.

Culture of Throat swab. In the laboratory the swab should be rubbed, while being rotated, over large ‘well’ areas, about one-third of the surface on each of two blood agar plates, and the wells should be streaked out with a loop over the remainder of the plate. The plates should be incubated at 37°C for 18-24 h, one in air plus 5-10% CO_2. the other anaerobically in nitrogen or hydrogen plus 5-10% CO_2. It is advantageous, before incubation, to place a 6 mm disk containing 1 unit of benzylpenicillin on the well area of one plate and a disk containing 0.1 units of bacitracin on that of the other.

Next day, colonies of S. pyogenes are recognized by their zones of β-haemolysis, larger and clearer on the anaerobic than the aerobic plate, and their sensitivity to both penicillin (zone diameter >16 mm) and bacitracin (zone >12 mm). Haemolytic Haemophili. which have streptococcus like, colonies, give stronger haemolysis on the aerobic than the anaerobic plate and are resistant to penicillin. The results of these primary sensitivity tests make possible the provisional identification of S. pyogenes, and its immediate reporting to the physician. even when the streptococci and their haemolysis are confined to the confluent mixed growth in the well of the plate and separate β- haemolysis colonies are not available for testing.

The bacitracin test fails to identify rare strains of S. pyogenes that are bacitracin-resistant and misidentifies rare strains of other streptococci that are bacitracin-sensitive. If, therefore well separated β-haemolytic colonies are present on the streaked-out area of the plate. they should be picked and their Lancefield group determined by a rapid co-agglutination or precipitation test . When separate colonies are not present. it is necessary to repeat the confluent (β-haemolytic growth to obtain a pure culture for grouping, but a report to the physician should not be delayed until the results of this confirmatory test are available.

Quantitative Culture. Note and report the relative abundance of S.pyogenes colonies in the primary plate culture, for the organism is more likely to have a pathogenic role when it is numerous (e.g. >100 colonies/plate) than when, it is scanty. An appreciable proportion of healthy persons, e.g. 1-10% of adults and up to 20 or 30% of children, carry the streptococcus in the throat, apparently without harm, and the organism will be detected in a throat swab when a carrier develops a sore throat due to some other pathogen, such as an undetected virus. Such a finding could lead to a misdiagnosis, and if the streptococci are scanty the possibility that they are not the cause of the pharyngitis should be borne in mind.

In a study of the value of standardized quantitative culture, it was found that large numbers of S. pyogenes in throat swabs from 71% of children with streptococcal pharyngitis, but in swabs from only 10% of well children who were throat carriers of the organism. But the numbers of streptococcal colonies on the culture plate are greatly influenced by the efficacy of the procedures of swabbing the throat and transfer to the plate, so that they may not fairly reflect the number of the organisms in vivo . As these procedures cannot be rigidly standardized. account should also be taken of the number of S. pyogenes colonies relative to the number of colonies of throat commensal bacteria

in the culture. Although a scanty growth of the streptococcus is likely to be due to harmless throat carriage, the uncertainty of the quantitative distinction makes it advisable that in all cases the organism's presence should be reported and antibiotics given to eradicate it.

Repots may be given as 'Many', Few' or No S. pyogenes found in culture'. When none is found. it is advisable to add to the report a statemert that 'Other pathogens, including viruses were not sought'. If a negative result is reported only as 'No pathogens isolated', the physician may think that the swab has been examined for viruses, Mycoplasmas, Chlamydias, Diphtheria bacilli, Vincent's organisms, Gonococci and other rarer throat pathogens.

Anti-streptolySin-O (ASO) titer. In cases of suspected streptococcal infection, e.g. acute rheumatic fever, where throat and other cultures have failed to reveal the organism, the patient's blood serum should be tested for its content of antibodies to streptolysin-O. In such infections there is usually a steep rise of ASO titer to values well in excess of 200 Todd unit/ml after 2-4 weeks. If the titer is not raised, the patient's illness is unlikely to be rheumatic fever. A commercial kit (Rapitex ASL) is available from Behring for rapid testing with a suspension al latex particles sensitized with streptolysin-O.

Other throat infections

Haemolytic streptococci other than S. pyogenes are often present in the throat as harmless commensals, but those of groups C and G occasionally, and B rarely, cause pharyngitis. If their presence in large numbers suggests they may have a pathogenic in the patient, their presence and antibiotic sensitivities should be reported to the physician.

Haemophilus influenzae. As haemophili are carried as commensals in the throat in a large proportion of adults and children, their finding in a throat swab should generally be ignored. There are, however, certain circumstances in which a search should be made for them and their presence regarded as possibly significant. Thus, ***H. influenzae*** of capsule serotype b is a fairly common pathogen in young children, especially under the age of 4, in whom it may cause pharyngitis,tracheo-laryngo-epiglottitis (croup). bronchopneumonia, bacteraemia and meningitis, and in such cases it should be sought in throat swab cultures. Moreover, in children with suspected bronchitis or pneumonia, it is often difficult to obtain a satisfactory specimen of sputum, and it may then be helpful to examine a throat swab for the presence of Haemophilus and other potential lung pathogens and determine their drug sensitivities. The physician should, however, be warned that though the pathogen in the throat may also be present in the lower respiratory tract, it is more probably confined to carriage in the throat. It may be difficult or hazardous to collect a throat swab from a child with croup, in which case an attempt should be made to demonstrate the pathogen in blood culture, as should also be done in suspected pneumonia and meningitis.

When Haemophilus is to be sought, the throat swab should be inoculated on to a heated-blood agar or Fildes agar plate as well as on to blood agar, and that plate incubated aerobically. A 2μg amoxycillin or ampicillin disk may be placed on the well of the plate so that both the presence and sensitivity of the haemophilus may be reported next day.

Until the ampicillin sensitivity is known, the drug of choice for the initial treatment of severe haemophilus infections is chloramphenicol, but its prolonged administration may be dangerous.

Diphtheria. In countries where diphtheria is even moderately common, all swabs from sore throats should be cultured on a selective tellurite indium for Corynebacterium diphtheriae and C. Ulcerans as well as on blood agar S. pyogenes.

Eleks test is used to detect exotoxin production from the isolated bacteria. It is composed of filter paper impregnated in antitoxin and put against the susbected culture.Precipitation lines will be formed around the filter paper

In communities where artificial immunization has made diphtheria rare, the chance of making a positive finding may be too low to justify the large expenditure of labor and materials in routinely setting up the extra cultures. In that case, reliance must be placed on the physician to indicate the few cases in which the possibility of diphtheria has been suggested by the presence of membrane in the throat, extreme constitutional upset or nerve paralyses, and which therefore require examination of the swab by the these methods.

Vincent's infection. A foetid, ulcerative inflammation of the throat (Vincent's angina) or gums (gingivitis) is occasionally caused by a combined infection with Vincent's spirochaetes and anaerobic fusiform bacilli. When the clinical findings suggest the condition, a swab from the affected areas should be examined in a Gram smear. The presence of many Gram-negative Spirochaetes and Fusiform bacilli, e.g. at least two of each per field well filled with pus cells and debris, may be reported as 'Many Vincent's organisms in film'. Small numbers of such organisms may be present in the healthy mouth and throat, and should be ignored.

Gonococcal pharyngitis. This condition should be suspected in promiscuous persons who engage in oral intercourse and a swab should be examined for gonococcus by culture on selective medium.

Candidiasis (thrush). In newborn babies and debilitated elderly persons, infection with ***Candida albicans*** may cause an acute inflammation with plaques of soft white exudates in the mouth and throat. in patients with these manifestations, a swab taken from the lesions should be examined for the fungus. An aerobic blood agar plate may show the small opaque white colonies of Candida, typically with short pointed 'rootlets' projecting from their margins, hut their growth may be slow and incubation at 35-37°C may have to be continued for 48h before they become recognizable. When an examination for candida is indicated. it is best to inoculate the swab on to a plate of Sabouraud agar as well as on to blood agar. A 50 unit nystatin disk and a 20µg amphotericin disk should be placed on the 'well' of the Sabouraud plate. Growth of candida sensitive to the antifungal drugs may then be observed and reported after 24 or 48h. and later be subjected to confirmatory tests. The presence of small numbers of yeasts in material from the mouth or throat may reflect selection and opportunistic colonization during antibiotic therapy. In such a case, further antibiotic therapy may be contraindicated.

Viral infections. Several viruses may cause an exudative pharyngitis resembling that caused by ***Streptococcus pyogenes.*** One that commonly. does so is the virus of infectious mononucleosis, a condition which may be diagnosed by the demonstration of a lymphocytosis and atypical lymphocytes in a blood film and that of heterophile antibodies in a Paul-Bunnell test on the patient's serum. Diagnostic tests are usually not attempted for other viral infections of the throat unless the identification is required for epidemiological purposes, when viral culture and serological diagnosis may be attempted.

Nasal, oral and sinus infections

The organisms infecting the nasal cavity are mainly the same as those infecting the throat and the two regions are often infected simultaneously. A deep nasal swab generally yields the same information as a throat swab. And it is usual to examine only the latter as a nasal swab seldom gives a positive result when the throat swab is negative. Nasal swabs are more often taken to detect healthy carriers than to diagnose infection, deep nasal swabs being taken kit for ***S. pyogenes*** and ***Diphtheria*** bacillus and swabs from the skin of the anterior nares for ***S. aureus***. Nasal carriers are a more dangerous source of infection for others than are throat carriers of the same organism, for they disseminate much larger numbers of organisms into the environment than the latter.

Stomatitis. Acute infections of the mouth are commonest in neonates and debilitated elderly persons. They can be caused by Vincent's organisms Candida albicans and Herpes simplex, Coxsackie A and various other Viruses. Swabs from the lesions are examined for the first two of these infections as described for throat swabs.

Sinusitis. The paranasal sinuses are normally sterile, but in the course of a nasal infection a nasopharyngeal bacterium such as H. influenzae or Pneumococcus may invade a sinus, most commonly the maxillary or frontal sinus, which then becomes filled with pus. Pus aspirated from the sinus, or a saline 'wash-out', should be examined in a Gram film and by culture on aerobic and anaerobic blood agar plates. Preferably the aerobic plate should be of heated blood agar.

Nasopharyngeal swabs. The collection of nasopharyngeal secretion by a pernasal swab in charcoal transport medium for the diagnosis of whooping-cough is described. A suitable pernasal swab kit with Amies charcoal transport medium is produced by Medical Wire.

Postnasal swab, in which the terminal 2 cm of wire bearing the swab is bent at an angle of 45° is passed behind the soft palate and rubbed on the posterior wall of the nasopharynx; it may be used for the detection of potential pathogens carried in the nasopharynx of healthy persons , e.g. Meningococcus which generally cannot be recovered from parts of the throat bathed with saliva from clue mouth.

Ear infection :

Swabs are taken from the external auditory meatus mainly in three suspected condition, acute otitis media, chronic suppurative otitis media and otitis externa. The provisional clinical diagnosis will indicate the different organisms likely to be present.

Acute Otitis Media. This infection is usually caused by S. Pyogenes, Pneumococcus, H. infuenzae, Branhlamella catarrhalis or, in many cases, One of the respiratory tract viruses . The organism spreads to the middle ear via the Eustachian tube from the nasopharynx which is the primary site of infection As the eardrum remains intact, none of the infected exudates can be collected on an ear swab, through culture of a throat swab may give a provisional indication of the causal organism. Antibiotic therapy is urgently required to prevent a possible bacterial infection damaging the hearing mechanism and amoxycillin erythromycin or cotrimoxazole may be used when the causal organism is unknown. Amoxycillin is the drug of choice unless a β-lactamase-producing variety of H. influenzae is the cause, When the absence of a rapid response will indicate the need for a change of drug.

If the eardrum has ruptured spontaneously, or a myringotomy has been performed to relieve pressure exudates may be collected on a thin swab introduced carefully into the external meatus. It should be examined in a Gram film and by aerobic and anaembic Culture plates of heated blood agar and blood agar.

Chronic suppurative otitis media When the eardrum has been perforated in an acute attack of otitis media and remains patent infection with the original pathogens may persist or repeated infections may be caused by secondary invaders such as S. aureus coliform bacilli, Pseudomonads and bacteroides. Swabs of the discharge in the external meatus should be cultured to guide the choice of antibiotics for systemic and topical therapy, but it must be borne in mind that such swabs are liable to be contaminated with commensal bacteria from the skin lining the meatus. These contaminants are mainly albus staphylococci diplitheroid bacilli and saprophytic mycobacteria which should be ignored but may include s. aureus and coliform bacilli.

Otitis externa. Chronic inflammation of the skin of the external meatus, with irritation and discharge, may be caused by bacteria, particularly ***Pseudomonas aerugjnosa colifoon bacilli and S. aureus,*** or fungi, most commonly ***Candida or Aspergillus***. A swab should be taken from the meatus and cultured aerobically on blood agar and MacConkey plates for the bacteria on a Sabouraud agar plate with a nystatin 50 Unit disk for 48h at 35-37°C for Candida and on a Sabouraud agar for 10 days at 28°C for Aspergillus . The results will guide the choice of drug for topical antibacterial or antifungal treatment.

Eye infections

Conjunctivitis and keratitis. The healthy conjunctiva and cornea usually bear a few albus staphylococci and diphtheroid bacilli, mainly derived from the edges of the eyelids. in the newborn, a severe form of acute conjunctivitis, ophthalmia neonatorum, may be caused by the gonococcus in the first 2 or 3 days of life and is liable to damage the cornea unless promptly treated with antibiotics. A much less dangerous infection. 'sticky eye'. may be caused by ***S. aureus*** during the first week or two. At any age, ***Haemophilus aegypzius*** may cause acute epidemic conjunctivitis and ***H. influenzae. Pneumococcus*** and ***Meningococcus*** may cause sporadic cases. ***Pseudornonas aeruginosa*** may cause serious superficial or deep infections after trauma or surgery to

the eye, and ***Moraxella Izicunata*** is found in a rare, subacute or chronic angular conjunctivitis.

Many cases of conjunctivitis are due to viruses of different kinds, e.g. ***adenovirus type 8*** which causes epidemic kerato-conjuctivitis in factories, shipyards and hospitals, whilst ***herpes simplex*** virus may cause keratitis. ***Chlamydia trachornatis*** causes trachoma, a common cause of corneal scarring and blindness in many undeveloped countries, and also a much milder, inclusion conjunctivitis in developed countries, e.g. swimming pool conjunctivitis in older subjects and congenital conjunctivitis within a few days of birth.

The principal difficulty in laboratory diagnosis is that of obtaining an adequate specimen in which the viability of the more delicate pathogens is preserved, it is best to make smears and seed culture plates beside the patient immediately after collecting, material from the eye.

Because the volume of exudates obtainable is generally small, a dry cotton-wool swab, which would absorb and retain most of the specimen, is unsuitable as a means of collection .

The exudates should be picked up with a sterile platinum loop or on the smoothly rounded tip of a thin glass or plastic rod; otherwise, on the tip of a thin, serum-coated swab. It should be collected from the conjunctiva, e.g. from under an everted eyelid, and contamination from the skin and margin of the eyelid should be avoided . A separate collection should be made for inoculation on to each culture plate and for the making of a smear. The cultures should be on blood agar and heated-blood agar plates incubated in air with 5-10% CO2.

When the specimen material is little, the smear should be confined to a small marked area of the slide, e.g. 5-10 mm in diameter; it should be stained by Gram's method with a strong counterstain.

If it is necessary to dispatch a specimen to the laboratory before inoculation on to Culture media, it should be taken on an albumen-coated swab which is placed at once in Stuart's transport medium.

For examination for chlamydia by immuno-fluorescence or culture in cells, scrapings must be taken from the affected conjunctIva after wiping off the exudate. For examination for viruses, a swab from the conjunctiva should be submitted in a virus transport medium.

Infections of orbit and eyeball. These may be caused by any of a variety of aerobic and anaerobic bacteria of the types found in pyogenic and wound infections. Any exudates obtainable should be examined for such organisms and a blood culture should be done. Iritis and choroidoretinitis may occur in the course of systemic viral infections, e.g. with cytomegalovirus, and toxoplasmosis, for which serological diagnosis should be attempted.

Styes are small boils affecting the follicles or the eyelashes on the edges of the eyelids; they are usually caused by ***S. aureus*** and treated without bacteriological investigation.

3. LOWER RESPIRATORY TNFECTIONS,

Unlike most regions of the upper respiratory tract the trachea, bronchi and lungs arc normally free from colonization with commensal and potentially pathogenic bacteria, but when their defenses are upset they are liable to be invaded by organisms from the throat. They are also susceptible to primary infection with various inhaled pathogens. such as the tubercle and whooping-cough bacilli, and to be involved in generalized infections such as measles and chicken-pox.

The commonest infections are acute tracheo-bronchitis, acute exacerbations of chronic bronchitis, and the pneumonias. in many or most cases the primary infection is caused by a virus, e.g. ***rhinovirus, myxovirus. adenovirus or respiratory syncytial virus,*** but there is often a secondary infection with a bacterial pathogen from the nasopharynx, most commonly ***Pneumococcus or Haemophilus influenzae. Pneumococcus*** also appears to be the primary cause of many cases of pneumonia, particularly lobar pneumonia, but often these pneumonic infections are triggered by a preceding viral infection of the upper respiratory tract, such as the common cold. Other secondary invaders of the lower tract include **Staphylococcus aureus**, which may cause fatal pneumonia after i***nflueanza, coliform bacilli and Pseudomonas aeruginosa, Branhamella cararrhalis, Candida albicans and Aspergillus fumigauss***. The ***staphylococcus, coliforms and candida*** are found particularly in hospitalized patients treated with antibiotics to which these organisms are resistant.

Other organisms that may cause primary infection in the bronchial tract or lungs are ***Mycoplasma pneumoniae,*** which is the commonest, ***Legionella pneumnophila***, ***Chlamydia psittaci B and Coxtella burnefi.*** The protozoon ***Pneumocystis carini*** is liable to cause diffuse infection of the lungs in persons who are immunosuppressed or immunodeficient, e.g. patients infected with human immunodeficiency virus.

Identification of a viral pathogen is attempted only occasionally, as when the information is required for epidemiological purposes or the diagnosis of an obscure infection. Laboratory diagnosis is employed mainly for the identification of other, particularly bacterial pathogens, which may be susceptible to treatment with an antibiotic chosen with the knowledge of their identity.

In suspected pneumonia and other severe infections it is usual to start antibiotic therapy without waiting for the results of laboratory tests, and as pneumococcus and haemophilus are the likeliest bacterial pathogens, the blindly chosen drug is generally ampicillin, amoxicillin, augmentin or cotrimoxazole. though erythromycin or tetracycline should be substituted if failure of response or the clinical features suggest that the infection may be due to a β-lactamase-producing haemophilus or a mycoplasma or legionella. and flucloxacillin should be added if S. aureus may be the cause. As the drugs so chosen may still be inappropriate. laboratory identification of the causal organism should always be attempted at the earliest possible stage. For the best chance of success, specimens of sputum and blood for culture should be collected before the start of any antibiotic therapy. In suspected atypical pneumonia an initial blood sample for serology should be taken at the same early stage.

Specimens

Sputum. The material from lower respiratory infections most commonly submitted for bacteriological examination is sputum, a mixture of bronchial secretion and inflammatory exudates coughed up into the mouth and expectorated. There are, however, difficulties both in collecting a suitable sample and in interpreting the results of its culture. In some infections, e.g. those due to Mycoplasma or Legionella, there is often a lack of secretion and sputum cannot be obtained.

Sputum from a bacterial infection is purulent, containing yellow or green opaque material as well as clear mucoid secretion. Staff collecting specimens should be instructed in how to obtain and recognize the correct material. Many patients tend to swallow their sputum when it is coughed into the throat and, when asked to spit some out, may expectorate mainly saliva. Saliva can be recognized because it is relatively clear and is watery rather than viscous.

Busy or uninstructed staff may send such collections of saliva to the laboratory, but they should not be examined, for the results are likely to be misleading. Thus, the specimen may fail to yield a culture pathogen in the lower tract and may give growth of an irrelevant potential pathogen that is carried in the throat. Any salivary specimen should therefore be discarded and a report send to the physician stating that the specimen was mainly saliva and thus unsuitable for examination. The regular practice of rejecting unsuitable specimens usually induces staff in wards and clinics to take greater care in the collection procedure.

The decision to reject specimens should not be left to junior staff and clear criteria for rejection should be laid down for general application. Preferably the criteria should be based on a microscopical as well as a naked-eye assessment. Thus, if a Gram-stained smear of a homogenized specimen shows less than 10 polymorphs to every squamous epitheial cell, and the patient is not leucopenic, the material probably consists mainly of saliva.

Instructions for collecting sputum should include the following advice.

1. Make the collection in a disposable, wide-mouthed screw-capped plastic container of about 100 ml capacity.
2. If possible collect the sputum before any antibiotic therapy is begun, and when the patient first coughs on waking in the morning.
3. Instruct the patient to wait until he feels materia1 coughed into his throat and then to work it forward into his mouth and spit it directly into the opened container, trying to avoid spilling over the rim. At once tightly screw on the cap of the container. Wipe off any spilled material on its outside with a tissue moistened with disinfectant, but take care not to let any disinfectant enter the container.
4. If the patient has difficulty in coughing sputum into his mouth, ask a physiotherapist to pummel his chest. This exercise often causes exudates to move in the bronchi and stimulate productive coughing.
5. Deliver the specimen to the laboratory as quickly as possible, preferably within 2h, for delicate pathogens such as Pneumococcus and Haemophilus may die out during any longer delay.

Bronchial swabs and aspirates. The principal difficulty in sputum examinations arises from the inevitable mixing of the expectorated specimen with throat secretion and saliva. It thus becomes contaminated with hardy mouth commensal bacteria that may overgrow the more delicate lung pathogens, and often also with potential lung pathogens, such as pneumococcus and haemophilus, which are commonly carried in the throat. When found, the latter may be wrongly thought to have been infecting the lower respiratory tract.

This confusing contamination can be avoided if a specimen of bronchial secretion is collected by some means that prevents its contact with the throat and mouth. Such collection may be done by transtracheal puncture and aspiration or by the use of a protected swab passed through a bronchoscope into the bronchi. Direct aspiration of secretion through a bronchoscope, e.g. by bronchial lavage, is unsatisfactory as the inside of the bronchoscope is liable to become soiled with throat secretion.

However transtracheal aspiration and bronchial swabbing require anaesthesia of the patient and the attention of skilled medical staff, and for these reasons are generally not performed. Nevertheless they may be attempted for the diagnosis of unusual or obscure infections.

Blood culture. In all cases of suspected pneumonia a sample of blood should be taken for culture before antibiotics are given. Lung infections are commonly associated with bacteriaemia and it may be possible to culture from the blood a delicate pathogen whose growth is suppressed in cultures of sputum contaminated with salivary organisms. Moreover, the finding of a bacterium in the blood is strong evidence that it has been infecting the lungs and is not merely a throat organism contaminating sputum.

Examination for tuberculosis

A policy must be decided for determining which specimens of sputum are to be examined for tubercle bacilli. Pulmonary tuberculosis is both a threat to the life of the patient and a dangerous source of infection to others. It is therefore that cases should be correctly diagnosed at the earliest possible stage and that drug therapy should be based on sensitivity tests made on a culture grown from the patient.

In communities where tuberculosis is moderately or very common, every specimen of sputum received in the laboratory should be screened for tubercle bacilli, regardless of whether the physician requests the examination. At least microscopy of a Ziehl-Neelsen or auramine-stained smear should be done and the positive specimens then cultured.

Dangerous suspected patients are all patients with unexplained cough continuing for more than 4-6 weeks, elderly persons with supposed ‘smoker’s cough’, coughing patients with haemoptysis or cachexia, persons who have been in close contact with patients diagnosed as tuberculous, and patients with illnesses that make them specially vulnerable (e.g. AIDS).

Laboratory staff must be protected against the risk of becoming infected from specimens containing tubercle bacilli. If, therefore, there nay be cases of pulmonary tuberculosis in the patient population served by the laboratory, all specimens of sputum should be regarded as possibly dangerous and the procedures of making smears and seeding cultures should be Performed in a safety cabinet.

Methods

-- Samples processing: All samples ARE subjected to decontamination by NaOH - NALC (N-acetyl-L-cysteine) method and then concentrated by centrifugation for 20 min at 10,000 g . Then the sediments were resuspended in sterile normal saline and subjected to Ziehl Neelsen (Z.N) smear and culture. Sterile samples like CSF, blood samples for Mycobacterium tuberculosis culture are not subjected to decontamination process.

- Loewenstein-Jensen (LJ) culture: Ready to use bottles of Loewenstein-Jensen media (Hispan Lab, Medicopharmatrade. Co, Madrid, Aspen) are inoculated with 0.1 ml of concentrated sputum sediments and will be kept in CO_2 incubator for 8 weeks. Bottles are inspected twice a week for visible colonies and suspicion growth is subjected to Z.N staining. Negative culture is discarded after 8 weeks (biohazard wate handling). Resulting growth is left in light for 2 hours and examined for yellow pigments to identify photo-chromogen species.

-Culture using radiometrie BACTEC 460 TB system: BACTEC 460 TB system and BACTEC 12B vials (Becton Dickinson Microbiology system, Cockeysville, Md.) are used for radiometrie culture of MTB . BACTEC 12B vials are prepared by addition of antimicrobial agents "PANTA" which contains polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin. One half ml of concentrated sputum sediments are inoculated into each BACTEC 12B vial and kept at 37°C and then tested 3 times a week. Positive cultures as indicated by BACTEC460 are subjected to further identification by NAP test (P-Nitro-acetyl amino hydroxyl-Proionophenone)for the presence of typical Myucobacterium tuberculosis.

-Antimicrobial Drug susceptibility testing (AST): Isolated TB colonies from BACTEC 12B media and from LJ media will be subjected to AST using both Agar proportion method and BACTEC 460 TB system .

a) Agar proportion method: The modified agar proportion method using Middlebrook 7H10 agar plates are used to determine the susceptibility of MTB isolates(Inderlied1996). Briefly, antibiotic stock solutions are diluted and added to Middlebrook 7H10 agar containing 10% oleic acid-albumin-dextrose (OADC) to give the following critical concentrations in quadrant plates: isoniazid (INH) 0.2 µg/ml; rifampicin (RIF) 1µg/ml; ethambutol (EMB) 5.0 µg/ml; streptomycin (SM) 2.0 µg/ml. One hundred microliter aliquots of diluted bacterial samples are inoculated to quadrants of drug containing or drug free media. Drug resistance in 7H10 method of proportion was defined as 1% or more growth of colonies on the drug containing agar quadrant compared to growth on the drug free quadrant. Results were recorded at 21 days after inoculation.

b) BACTEC 460 TB system: The recommendation of the manufacture for sensitivity testing, the modified critical concentrations of provided drugs (SIRE, Becton Dickinson) are adopted: 2 µg/ml for SM; 0.1 µg/ml for INH; 2.0 µg/ml for RIF; and 2.5 µg/ml for EMB.

-Bacteriophage methods or PCR using primers for the target sequences.

-Direct PCR to detect Mycobacterium tuberculosis in clinical samples.

-Tuberculin skin test. Inject 5-10 Iu/ml PPD reagent intradermal in forearm by insulin syringe an surround the area of injection by pen. Read result after 24-48 hours. Interpretate positive if diameter if induration is 10mm or more.

Examination for other Respiratory common infections

All sputa should be examined for Pneumococcus, Haemophilus and the other aerobic pathogens that commonly infect the bronchi and lungs. The recommended procedures are as follows.

1) Note whether the specimen contains opaque green-yellow pus. Do not examine specimens consisting of clear. watery saliva.

2) Homogenization. There are advantages in homogenizing the specimen before making films and cultures. Most sputa are inhomogeneous. The purulent material, which contains most of the relevant pathogens, is usually embedded in clear mucoid secretion, and if the specimen is not first homogenized it is difficult to separate out a purulent portion for filming and culture. If the specimen is homogenized, every drop and loopful of it will contain some of the pathogens present. Moreover, the homogenized material is suitable for quantitative examinations.

To homogenize, mix and incubate equal volumes of the sputum and a solution of dithiothreitol (e.g. Sputolysin, Calbiochem) or buffered pancreatin (Oxoid) made up according to the manufacturer's instructions. With dithio-threitol, either mix rapidly on a vortex mixer for 15 s and stand for 15 mm at ambient temperature or, preferably, mix gently and continuously on a machine that tilts to and fro placed in an incubator for 30 mm at 37°C. With pancreatin, incubate for 30 mm at 37°C with continuous or occasional shaking.

3) Microscopy. Make a smear of the homogenized sputum, or a purulent portion of the sputum if it is not homogenized. Stain by Gram's method and examine with oil-immersion. First note the presence and relative numbers of polymorphs and squamous epithelial cells. If there are less than 10 polymorphs per squame, the specimen is probably mainly saliva; if more, it is probably derived from an infected site in the lower respiratory tract.

Next note whether there is a wide diversity of bacterial forms, suggesting salivary contamination, or the predominance of one potentially pathogenic form, e.g. Gram positive diplococci (probably pneumococci), small slender Gram-negative (probably haemophili), or clustered Gram-positive cocci (probably Staphylococcus aureus). Any of the latter three findings warrants the phoning of a provisional report to the physician to guide his initial choice of antibiotics for therapy. The finding of numerous staphylococci is particularly significant, as it indicates that treatment with a β-lactamaseresistant penicillin, such as flucloxacillin, is urgently required.

If fungal infection, e.g. Aspergillosis, is suspected, a wet film should be carefully searched for the presence of conidiophores. The presence of such sporing heads indicates that the fungus is growing in the bronchial tract, whilst the observation of a few colonies growing in a culture of the sputum may reflect only the recent inhalation of spores from the environment.

4) Culture. A semi-quantitative method of culture is recommended, so that the presence of a potential pathogen in only small numbers, e.g. less than 106/ml sputum, may either be ignored or be reported to the physician as probably representing contamination of the specimen from the throat. If, however, antibiotic treatment had been started before the specimen was taken, or if special considerations apply. as in cystic fibrosis, the presence of a potential pathogen in small numbers should not be ignored.

A satisfactory procedure is to dilute the 1-in-2 homogenized sputum a further 1 in 100 in sterile broth and to inoculate a 0.005 ml loopful of the dilution on to each culture plate.

The inoculum's should be spread co fluently over half of the plate and streaked out over the other half. The growth on the whole area of the plate of 25 or more colonies of the same potential pathogen will then indicate that 106 or more of that pathogen were present in each milliliter of the original sputum. The cultures should be incubated at 37oC for 18-24 h in humid air plus 5-10% CO2 and re-incubated if the colonies are then still small and indistimet.

If lung or bronchiectasis is suspected, need additional plates be set up for anaerobic incubation, which should be for 2-4 days. However a few strains of pneumococci appear to grow in primary cultures only under anaerobic conditions. so it may be best to culture all specimens anaerobically on an extra blood agar plate having a 5µg optochin disk placed on a confluently seeded area and examine this after 18-24 h.

A plate of good blood agar should suffice to give characteristic growth of the main pathogens but it is preferable also to seed a plate of either heated-blood ('chocolate') agar. Which may give better growth of pneumococcus and haemophilus, or Fildes digest agar, which gives a very distinctive growth of H. influenzae and tends to suppress the growth of many salivary commensal bacteria.

5) Identification tests. If, before incubation, of the primary cultures, a 5µg optochin disk and a 1 unit benzylpenicillin disk are placed on the confluently seeded area of the blood plate and a 2µg amoxycillin disk on that of the Fildes plate, the identity and sensitivities of pneumococcus and haemophilus isolates may be read and reported the day after receipt of the specimen. Further identification and sensitivity tests may be considered unnecessary, but care should be taken not to overlook. β-lactamase production in a haemophilus.

Staphylococcal isolates should be tested by the coagulase or DNase test for identification. of S. aureus and tested for drug sensitivities, particularly to benzylpenicillin and a β-lactamase-resistant penicillin, such as methicillin. Pseudomonas should be identified by an oxidase test performed on a colony on the primary plate, and tested for sensitivity to antipseudomonas drugs. It is seldom necessary to identify other coliforsns, but if one is numerous and thought possibly to be of pathogenic significance, not merely an overgrowing contaminant, its antibiotic sensitivities should be tested. Any numerous Neisseria-like colonies should be tested by a rapid method for the fermentation of glucose, maltose and sucrose to identify Branhamella catarrhalis, which does not ferment them.

6) Fungal culture . Candida may be recognized as small opaque cream-coloured colonies with spiky projections on blood agar. These should be subcultured for tests of sensitivity to nystatin and amphotericin and germ-tube formation (C. albicans). If. however, the request form indicates that a candidal or fungal infection is suspected, the homogenized sputum should be seeded on to a plate of Sabouraud or ma1t extract agar with a 50 unit nystatin disk for aerobic incubation at 35-37°C for 2 days for the culture of candida, and a slope of Sabouraad or malt extract agar for aerobic incubation at 28°C for 10 days.

Pneumocystis pneumonia. In an immunosuppressed or immunodeficient patient with signs of pneumonia or diffuse infiltration of the lungs. infection with Pneumocystis carinii must be considered and its diagnosis made quickly, for treatment with cotrimoxazole or pentamidine is required for this often fatal condition.

The diagnosis depends on the microscopical demonstration of the Gram-negative trophozoites (1-2 μm) or the more easily seen Gram-positive cysts (c. 5 μm) of the protozoon. Sputum, or a tracheal or bronchial aspirate rarely gives a positive result. A specimen of lung tissue should be obtained by peicutaneous needle aspiration or open lung biopsy or one of alveolar fluid by bronchoscopic alveolar lavage.

Press the cut surface of tissue on a slide to yield an "imprint" without smearing. Centrifuge lavage fluid and spread about 0.1 ml of the deposit as a thick film on a slide. Dry the imprint or film and fix with brief heating. Stain the film by the Grocott methenamine silver nitrate method (takinig c.3h), its rapid (5 mm). The silver stains the thin wall and two internal comma-shaped bodies of the cysts black, and their cytoplasm a light golden colour, but does not Stain the trophozoites. The cysts are commonly seen in clusters embedded in amorphous debris. Care must be taken to distinguish them from yeasts, which usually stain solidly and may show budding. A second film may be stained by the Gram-Weigert method. Which colors wall and internal bodies of the cysts purple-black.

Rarer bronchopulmonary infections When suspected, infections with Mycoplasma. Legionella, Chlamydia, Coxiella and viruses may be diagnosed by culture on appropriate media if available or by PCR. Serological tests can be helpful.

Croups It is an acute infection that affects the upper and lower respiratory tracts, especially the larynx, trachea, and bronchi, and is caused most commonly by viruses of the genus Paramyxovirus and repiratory syncitial virus. Bacterial Croups results from bacterial tracheitis, Eppiglositis and bacterial tracheitis. It leads to inflammation and infection of the upper trachea, swelling inflamed tissues, likely began as viral or other preexisting lesion, develops into bacterial super-infection . It is characterized by labored breathing and obstruction below the glottis, accompanied by a barking cough. The coomon age from 1month - 13yrs, gaurded prognosis(untreated will not do well), progression from URI. Clinical manifestations are high fever, initial croupy cough, progressive stridor, airway compromise, purulent (pus) secretions , general lethargy. Diagnosis is confirmed by blood culture. Treatment items include antibiotics for staphylococcus, IV fluids, oxygen nursing care focus on airway assessment and keep secretions liquefied and keep oxygen saturations greater than 93%.

4) WOUND, SKIN AND DEEP SEPSIS

This section deals with the diagnosis of mainly suppurative infections of wounds, burns, the skin, ulcers and sinuses, sites that are open to contamination with more than one of a variety of organisms, from the skin, respiratory tract. alimentary tract or the environment; also of closed abscesses and other infections of deep sites, e.g. osteomyelitis and septic arthritis, usually infected by only a single species of pathogen.

Wound infections may be endogenous or exogeuous. Endogenous infections, or auto-infections, are caused by organisms that have been leading a commensal existence elsewhere in the patient's body; for example, an abdominal surgical wound may become infected with organisms from the large bowel after an operation involving incision of the colon. In exogenous infections the source of the infecting organism is out the body of the patient who becomes infected; cross-infection is a particular example of exogenous infection in which the causal organism is spread from person to person. Infection may occur after accidental or intentional trauma of the skin or other tissue; the latter type is often called "surgical" or "postoperative sepsis".

Infection of a wound is difficult to define and no clear rules can be given to distinguish it from contamination and colonization. Wounds and other open lesions are liable to contamination with a multiplicity of organisms from the body surfaces and environment; the contaminating organisms are at first generally present in relatively small numbers, as originally introduced, and need not subsequently multiply. Infection occurs when one or more of the contaminants

Evades the clearing effect of the host's defenses, replicates in large numbers and attacks and harms the host's tissues. In the case of a commensal or low-grade pathogen. the multiplication may cause little or no harm to the host and may best be described as colonization. Whether harmful infection or harmless colonization occurs is dependent on the virulence of the organisms and the local and general resistance of the host. A knowledge of the patient's general and local condition is therefore important in assessing the significance of bacteriological findings.

Infections of soft tissues are generally associated with the production of pus and the bacteria involved are said to be 'pyogenic' (pus-producing).

As there is a wide range of possible causative organisms, a degree of selectivity is inevitable in the choice of examination procedures in the laboratory. The physician has a responsibility to guide the search by noting clues to the nature of the infection in the patient's history and clinical appearance. In many cases the likely cause is fairly obvious. There are well recognized associations of Staphylococcus aureus with pustules, boils, carbuncles, stitch abscesses and wound infections. Similarly, Streptococcus pyogenes is classically associated with the spreading lesion of erysipelas, which may present as a fulminating infection. However, such associations are not invariable, and vigilance and awareness are essential.

A wide variety of aerobic and anaerobic species of bacteria may be present, either singly or in combination, in infections of wounds and other soft tissues. The commonest pyogenic bacteria are S. aureus, S. pyogenes, Pneumococcus and coliform bacilli such as Escherichia coil, Proteus species and Pseudoinonas aeruginosa. Anaerobic

organisms, particularly Clostridium perfringens and other clostridia, Bacteroides species and anaerobic cocci, may be important in infections of wounds, especially abdominal wounds, soiled deep or lacerated wounds, and wherever devitalized tissues provide suitably anaerobic condition preliminary infection with aerobic bacteria, by consuming the available oxygen. may encourage the growth of clostridia and lead to gas-gangrene or tetanus .

In many cases there is a mixed infection with more than one bacterial species, and in some of these cases a pathogenic synergy may be evident with two or more species acting in concert to cause more damage to the tissues than would be caused by either alone. Mixed infection with Gram-positive cocci and coliform bacilli are not uncommon and polymicrobial infections. With anaerobes such as bacteroides and fusiforms, or fuso-spirochaetal associations are well recognized. In Vincent's infection of the gums, for example. and in various other fuso-spirochaetal conditions, the mixture of species may be associated with a fulminating attack.

Special associations of certain pathogens with particular conditions should be borne in mind, e.g. those of bacieroides and anaerobic cocci with dental and cerebral abscesses, capnophilic cocci with dental, cerobral and hepatic abscesses, and anaerobes with pelvic inflammatory diseases. Many postoperative abdominal or pelvic wounds have coliform bacilli associated with a moderate. exudate during the early healing stage, the infection being often superficial and resolving without specific therapy. But a combination of coliforms with bacteroides may cause a more severe, synergic infection calling for prompt antibacterial therapy.

Among species much less commonly encountered are Pasteurella multocida in animal bites, Cornebacterium diphtheriae in wound diphtheria, and Bacillus anthracis in malignant pustule of the skin. In chronic infections that are slow to heal and in pus showing no other microbes, the possibility of infection with Mycobacterium tuberculosis, other Mycobacteria, Actinomyces israeli, nocardiac or fungi must be considered.

Collection of specimens

Pus or exudates is often submitted on a swab for laboratory investigation. The swab is an inefficient sampling device and tends to desiccate the specimen and trap the bacteria, which are then not released on to the culture plate. Whenever possible. pus or exudate should be submitted in a small screw-capped bottle, a firmly stoppered tube or syringe, or a sealed capillary tube. Some workers advocate the sending of pus in oxygen-free, gassed-out vials or in special transport media. We recommend the routine use of transport medium swab kits where delay in transit is likely, as from general practice patients.

Fragments of excised tissue removed at wound toilet or curettings from infected sinuses and other tissues, should be sent in a sterile container without fixative. They are homogenized in a tissue grinder with a little sterile broth and subsequently treated in the same way as exudates.

Delay in the transit of specimens to the laboratory must be avoided, especially in the case of swabs, where the exudate may dry into the cotton-wool.

If it is decided to compromise and send a swab, load the swab well with the material. If possible, send two swabs taken from the depths of the wound or lesion, so that one can be used for the preparation of a smear for microscopy and the other for the seeding of cultures. It is difficult to make a good smear from a swab, and quite impossible if the swab is dry; a dry swab must first be moistened with a little sterile broth or saline, and even then the smear may be inadequate. To avoid contamination of the swab before it is used to seed the cultures, either it c must be smeared on a sterilized slide or else used to make the smear only after it has been seeded on to all the culture media. The latter procedure is Often followed and a poor, unrepresentative smear is usually the result. The submission of duplicate swabs avoids this difficulty. The examination of material on swabs for mycobacteria is almost always unsatisfactory.

If it can be arranged, an extra swab should be placed and broken into a cooked-meat broth Immediately it has been taken from the lesion in the ward or at operation, and this broth Containing the swab head should be sent to the laboratory in addition to two conventionally packaged swabs. Pathogens sometimes grow in the directly seeded broth, and in subculture from it, when they cannot be recovered in culture from the conventional swabs.

Physicians should be instructed that when a special investigation is required. they should state this clearly on the request form. Thus the routine investigation is usually confined to a search for the common pyogenic bacteria and anaerobic pathogens and does not include an examination for the tubercle bacillus, other Mcobacteria, Actinomyces, Nocardia, the Diphtheria bacillus, the Anthrax bacillus or fungi.

Blood culture. If the patient is febrile or in shock, or it seems possible that his local infection is accompanied by a bacteriaemia, a sample of blood should be taken for culture.

Laboratory examination

The basic procedures usually include a naked-eye examination of the specimen, the microscopical examination of a Gram film, and culture on aerobic and anaerobic blood agar plates, on MacConkey agar and in cooked-meat broth. Gas chromatography may be performed directly on liquid specimens to indicate the presence of anaerobes.

Naked-eye examination

The appearance of a specimen of pus or exudate, and that of any appreciable amount of pus on a swab, should be noted on initial examination.

The pus of a staphylococcal lesion is typically creamy and thick in consistency, with pus cells evident on microscopy. That of a Streptococci pyogenes infection is generally straw-coloured and, watery, with lysis of pus cells on microscopy. That of proteus infection has a fishy small and that of pseudomonas infection a sweet, must odour and often a blue pigmentation.

Pus containing anaerobic organisms often has an offensive putrid small, and that of actinomycosis often contains small microcolonies that appear as "sulphur granules". In some fungal infections such as mycetoma. black or brown granules may be present. The pus of an amoebic abscess is said to resemble anchovy sauce .

Microscopy

Much useful information may be obtained from a smear stained by Gram's method. First note the presence and relative numbers of polymorphs and bacteria. Pay particular attention to the numbers and variety of different morphological forms of Gram-positive and Gram-negative bacteria. If in a serious infection the appearances suggest the presence of a particular pathogen, a provisional report should be telephoned to the physician to guide his initial treatment of the patient. Gram-positive cocci in typical dusters or chains may suggest a staphylococcal or streptococcal infection, but care should be taken, as the sheared edge of a cluster of cocci may simulate a chain of streptococci and a tangled streptococcal chain may simulate a staphylococcal cluster; mixed staphylococcal and streptococcal infections also occur. The appearance of Gram-positive diplococci may be given by either pneumococci or enterococci. Faintly staining Gram-negative rods are sometimes missed if much background debris is heavily counterstained. Gram-variable filaments of actinomyces may appear like chains of cocci and their fragments as diphtheroid bacilli. Many Gram-positive clostridia appear as Gram-negative forms in pus.

Examination of a wet film may reveal the presence of fungi or motile bacteria and fluids aspirated from inflamed joints resembling septic arthritis should be examined in a wet film for the presence of uric acid crystals, which may be responsible for the condition in the absence of any infection. Darkground microscopy of a wet film is useful in the diagnosis of primary syphilis .

A smear stained by the Ziehi-Neelsen rnethod should be examined when the clinical circumstances suggest that the tubercle bacillus another mycobacterium or a nocardia may be present, e.g. in chronic and neck abscesses. Immunotluorescent staining allows the prompt identification of pathogens for which specific antisera are available, e.g. some pathogenic clostridia.

Culture

The specimen should be inoculated on to plates of blood agar, the one for incubation at 37°C aerobically, preferably in air plus 5-10% CO2. the other for incubation anaerobically in nitrogen/hydrogen plus 5-10% CO2. It should also be plated for aerobic incubation on MacConkey agar or CLED agar for the differentiation of coliforms staphylococci and enterococci, and be inoculated into a tube of cooked-meat broth for the enrichment of exacting aerobes and anaerobes. If the specimen is a scantily charged swab; it should be inoculated first on to the blood agar plates, then on to the MacConkey, and finally. into the cooked-meat broth, where it should b soaked in the broth and squeezed out on the inside wall of the tube several times.

If there is reason to expect the presence of a spreading organism such as proteus, which might overgrow to obscure the isolation of other pathogens, the specimen should also be inoculated on to a plate of a medium that inhibits spreading, e.g. PNPG blood agar containing 0.43 g p-nitrophenylglycerol per litre or blood agar containing two to three times the usual concentration of agar. If the firm agar is to be used, the bacteriologist should have familiarized himself with the small, often atypical appearance of the colonies of the common pathogens grown on it. Spreading of proteus

is inhibited on MacConkey agar, but the use of that medium is insufficient as some common pathogens, such as Streptococcus pyogenes, fad to grow on it.

It is a useful practice to place one or two anti biotic disks on the 'well' areas of the blood agar plates before incubation e.g a 1 unit benzyl-penicillin disk and a 10 µg gentamicin disk on the aerobic plate and a 5µg metronidazole disk and a 50µg neomycin disk on the anaerobic plate. These 'primary sensitivity tests' may give an early indication of an important aspect of antibiotic sensitivity, assist in the identification of colonies of species with constant sensitivities. and facilitate the isolation of resistant species from mixed cultures by the picking of colonies from the inhibition zones. Thus, strict anaerobes but not aerobes or facultative organisms are inhibited by metronidazole, whilst many anaerobes are resistant to neomycin which inhibits most aerobes. If the appearances in the Gram smear suggest the extra procedures will be worthwhile, a full set of antibiotic disks may be used on a separate plate seeded heavily and confluently from the specimen.

The culture plates are examined after overnight incubation at 37°C for 18-24h, when the relative numbers and types of the colonies should be noted and any further tests required for their identification and determination of their antibiotic sensitivities done. If there is no growth on the plates, or no growth of a type of organism seen in the Gram film, the aerobic and anaerobic blood agars should be reincubated for another 24h. If there is still no growth, the plates may be discarded unless there is an indication for longer incubation, as for 7 days when the presence is suspected of a slow-growing pathogen such as Actinomyces israeli or some species of bacteroides. If at 24h or 48 h there is growth in the cooked-meat broth, indicated by turbidity in its supernate, but no growth on the plates, the broth should be filmed and subcultured, both aerobically and anaerobically.

There is a difficulty relating to the culture of slow-growing anaerobes that are highly sensitive to killing by oxygen. Their still invisible growth may be killed by the exposure to air when the anaerobically incubated plate is opened and examined on the bench at 18-24h, and so not develop further when the plate is reincubated anaerobically. It is important therefore to return the plates to an anaerobic atmosphere as quickly as possible after their opening and examination, but when many specimens ate being examined it may be difficult to avoid exposure to air for up to an hour or more. There are thus advantages in continuous anaerobic incubation of primary culture plates. This may be done by the use of an anaerobic cabinet in which the plates are incubated continuously, but may be inspected at intervals through a window without being removed from the anaerobic atmosphere.

Otherwise, the specimen may be inoculated on to two anaerobic plates, the one to be removed for examination after 18-24 h, the other to be left undisturbed in a separate anaerobic atmosphere for later examination after 4-5 days. This second procedure requires the availability of large numbers of anaerobic jars.

If tuberculous or fungal infection is suspected, whether from the clinical circumstances, the appearances in a film, or the absence of growth of bacteria on the

ordinary media, the specimen should be cultured by the appropriate methods on the appropriate special media.

Identification of isolates

After the bacteria cultured have been obtained in pure subcultures, any further necessary tests for their identification should be done, e.g. the coagulase test on staphylococci. Lancefield's grouping of β-haemolytic streptococci. and biochemical tests on coliform bacilli and anaerobes. At the same time the pure cultures should be tested for sensitivity to an extended range of antibiotics useful in therapy. When mixed bacteria are grown from a wound or other superficial lesion liable to be contaminated with commensal and saprophytic bacteria, the isolates judged to be such harmless contaminants, e.g. albus (coagulase-negative) staphylococci, diphtheroid bacilli and aerobic spore-formers, need not be fully identified or tested for antibiotic sensitivities.

interpretation and reporting

A pure growth of a recognized pathogen obtained from a wound or closed abscess is easily interpreted as significant and will be reported to the physician as being so. Difficulties arise in the interpretation of mixed cultures grown from superficial lesions contaminated with commensal and saprophytic organisms.

Scanty growths of skin commensals such as staphylococci albus and diphtheroid are usually disregarded and not reported, and similarly a few colonies of Escherichia coli growth from a perineal or other wound liable to be contaminated with faecal bacteria. If Potentially dangerous faecal commensal such as Clostridiuin perfringens is isolated in smal1 numbers under such circumstance, the physician should be made aware of the potential danger while being advised of the probability that the organism is present only as a contaminant. It is particularly in chronic superficial lesions. such as varicose ulcers, that the presence of mixed commensal bacteria can confidently be disregarded as insignificant. The result may then be reported on the following lines, "Many mixed faecal and skin bacteria present", without giving identities or antibiotic sensitivities. But a pure growth of a commensal-type bacterium grown from a specimen aspirated from a deep site not subject to contamination, e.g. joint or pleural fluid, should be reported. together with sensitivities, unless the number of the organisms is so small as to indicate they are contaminants picked up during collection of the specimen or inoculation into the media.

Some indication of the importance of the different bacteria cultured from a lesion may be seen in the number and proportion of their forms in the Gram film and their colonies on the primary plates. In general, a numerous or predominant organism is likely to have pathogenic significance, but the relative numbers of the colonies of the different organisms on a culture plate may not reflect the relative numbers of the organisms in the lesion, for they are subject to many variations such as, the relative speed of growth of the different species under the cultural conditions used, antibiotic interactions between species, the presence of traces of antibacterial drugs in the specimen, and the greater tendency of the more delicate pathogens, such as Pneumococcus, Haemophilus and anaerobes, to die during transport of the specimen to the laboratory. For such reasons, a causal pathogen may be cultured in, smaller numbers than a contaminating commensal.

If must be borne in mind that the number of colonies grown in a subculture from a primary enrichment culture of the specimen in cooked meat broth bears no relation whatever to the number of organisms in the lesion. Even a single bacterium inoculated into the broth may yield a profuse culture in 18 h. When, however , the broth shows growth of an organism not found on the culture plates, it may be concluded that probably only very small numbers of that organism were present in the specimen . Thus when a growth of Staphylococcus epidermidis is obtained in the broth but not on the plates, it is likely the organism was present only as a scanty contaminant of the specimen.

Sometimes the significance of a growth is difficult to assess in the laboratory, but the problem may often be resolved by discussion with the physician and examination of a further. Carefully taken specimen from the lesion.

Fluid aspirates

Aspirated specimens of joint, pleural, pericardial and peritoneal fluids are examined generally as described above, but if the fluid is relatively clear and its volume sufficient, it should first be centrifuged to deposit the cells and bacteria, and the supernatant discarded into disinfectant. As nutritionally exacting pathogens, such as Pneumococcus, Haemophilus and gonococcus may be present, an extra plate of heated-blood ('chocolate') agar should be seeded for incubation in air plus 5-10% CO2. Deposits from joint and pleural fluids not yielding other bacteria should be examined for tubercle bacilli by Ziehl Neelsen film, culture on Löwenstein-Jensen medium and Bactec media.

Peritoneal dialysis

Many patients with renal failure are now treated by the procedure of peritoneal dialysis which exposes them to the risk of bacteria being introduced into the peritoneum and causing a Serious peritonitis. Laboratory examination of the effluent fluid is required so that the infection may be recognized at the earliest possible stage and appropriate antibiotic therapy given. When the effluent fluid is turbid to the naked eye, it is obvious that infection has occurred , but examination by cell count, Gram film and culture may reveal the development of the infection at an earlier stage as well as identifying the infecting organism.

Most infections are caused by coagulase negative staphylococci, usually Staphylococcus epidermidis derived from the skin, but a few are caused by coliform bacilli, enterococci, viridans streptococci. S. aureus, diphrheroid bacilli. candida and anaerobic bacilli. Until the identify and sensitivities of the causal organism are known, the infection may be treated with antibiotics. e.g. cefuroxime, gentamicin, added to the dialysis fluid.

The whole bag of effluent should be delivered to the laboratory, where there are the skilled staff and facilities to remove a sample without introducing contaminating bacteria. The clip on the spout of the bag should be loosened, a little fluid run to waste, and then about 200 ml fluid collected in four 50 ml lots in sterile bottles suitable for centrifuging.

Before centrifugation, a little of the mixed fluid should be taken for a leucocyte count, which is done in a counting chamber as used for cerebrospinal fluid cell counts. In the

absence of infection the fluid will contain 10, 20 or 30 leucocytes/m1, but in peritonitis often 500/ml, 1000/mI or more. A count higher than 100/ml should arouse the suspicion of a developing infection. The presence of eosinophilic polymorphs may suggest that the patient's symptoms are due to hypersensitivity to components of the dialysis fluid rather than to infection.

The 200 ml of fluid should then be centrifuged, the supernatants discarded and the cellular deposits pooled in about 20 ml of residual fluid. This suspension should be used to make a film for Gram staining and several drops of it should be inoculated on to blood agar plates for aerobic and anaerobic incubation, and a MacConkey plate for aerobic incubation. The remainder of the deposit should be added to a tube of cooked-meat broth. The plates are examined after 18-24 h and again at 48 h. If they show no growth the cooked-meat broth is incubated for 3 weeks, or until it becomes turbid, when It Should be examined by film and subculture. An alternative approach is to inoculate samples the fluid into a standard set of blood culture bottles in the place of the cooked-meat broth; the bottles are then incubated and subcultured by the standard procedures. Any organism isolated is identified and its sensitivities are determined. If the leucocyte count is raised. or if bacteria are seen in the Gram film or in culture, the finding should immediately be telephoned to the physician without waiting for identification of the bacteria.

5. GENITAL TRACT INFECTIONS

The laboratory approach to the diagnosis of genital tract infections is best considered in relation to the sex of the patient. Although some of the specific infections, e.g. gonorrhoea, syphilis and chlamydial infection, are common to both sexes, there are usually differences in the presenting symptoms and the sites and methods of collection of specimens in these infections. Moreover, some other infections, e.g. vaginitis and uterine sepsis, are confined to women.

Genital infections in women

These include urethntis, vaginitis, vaginosis, genital ulceration, cervicitis, uterine sepsis, salpingitis, oophoritis and the condition recognized as pelvic inflammatory disease.

Vaginal discharge. Excessive vaginal discharge which is purulent or normal in character is a common complaint, especially in sexually active women, and accounts for a large proportion of specimens submitted for laboratory examination for genital tract infection.

A wide range of organisms may be associated with leucorrhoea, but the roles of some are still uncertain. In acute vaginitis the squamous epithelial lining of the vaginal wall is invaded and inflamed, causing discomfort, pruritis or pain in addition to discharge. It is most commonly caused by Trichomonas vaginalis or Candida albicans or other yeasts such as Torulopsis glabrata. Another common condition is anaerobic vaginosis, in which the vaginal epithelium is not invaded or inflamed and the main presenting symptom is the presence of a putrid or fishy-smeil1flg discharge. It appears to be caused by the excessive growth of a mixture of bacteria, including anaerobes, that are commonly present in smaller and harmless numbers in the healthy vagina, e.g. Gardnerella vaginalia Bacteroides species, anaerobic cocci and anaerobic vibrios (mobiluncus). The value of its diagnosis in the laboratory is that it often responds to treatment with metronidazole .

Cervicitis with or without urethritis may be caused by infection with the gonococcus or Chlamydia trachomatis and may lead to vaginal discharge. In gonorrhoea there is also often infection of Bartholin's glands, but only rarely infection of the vaginal epitbelium. Chlamydia infection may occur with minimal signs and symptoms. Infection of the uterine tubes (salpingitis) with gonococci, streptococci or coliform bacilli may or may not be accompanied by vaginal discharge.

Uterine sepsis. either puerperal or postabortion. may or may not lead to an increased or abnormal discharge of lochia from the vagina, and the main clue to its diagnosis is the onset of pyrexia. Streptococcus pyogenes is the classical cause of puerperal uterine infection, which may lead to fatal septicaemia. Streptococci of other Lancefield groups, anaerobic cocci, Staphylococcus aureus, coliform bacilli, bacteroides and clostridia are sometimes involved, either singly or in combination. These organisms may also be involved in postoperative infection of the genital tract, which may proceed to a life-threatening peritonitis or septicaemia.

Genital ulceration may be caused by herpes simplex virus, notably type 2, *Treponema pallidum, Haemophilus ducreyi, Chlamydia* of group A, and

Corneybacterium granulomatis. Pathologists and clinicians recognize abnormal states of the epithelium of the uterine cervix variously described as cervical ulceration erosion, metaplasia and dysplasia. There have been debated associations of herpes simplex virus type 2 (HSV2) with these Conditions and possible neoplastic change, and more recently a link with certain human papilloma viruses (HPV) or a combined effect of HSV2 and HPV has been suggested. The cervix should be sampled directly when genital herpes is suspected in a pregnant woman.

Tuberculous infection of the uterus and uterine tubes may be caused by Mycobacterium tuberculosis, M. bovis or one of the other pathogenic mycobacteria. Actinomycotic infection of the uterus may be associated with the use of an intra-uterine device. The toxic shock syndrome is attributed to the elaboration of a potent exotoxin by staphylococci contaminating and growing on vaginal tampons.

Commensal flora. In relation to the collection of specimens and the interpretation of 1aboratory findings, it must be borne in mind that the vulva and urethral meatus are contaminated with skin, faecal and vaginal organisms, that the vagina has an indigenous commensal flora, and that the uterus, uterine tubes and ovaries are normally free from microorganisms. It is only for a few days after abortion or childbirth that bacteria from the vagina or environment are likely to piss into the uterus, though gonococci, pneumococci and other organisms occasionally cause ascending infections under other circumstances.

The commensal flora of the vagina varies with age. Before puberty, when the secretion is not acid (pH 6.5-7.5), the flora consists mainly of staphylococci, streptococci other than S. pyogenes, diphtheroid bacilli and coliforms. From puberty to the menopause, the secretion is acid (e.g pH 4.5) and lactobacilli predominate, though various other organisms are present in smaller numbers, e.g. staphylococci, enterococci, Streptococci other than S. pyogenes, diphtheroid bacilli, coliform bacilli, bacteroides, anaerobic cocci, anaerobic vibrios, yeasts and mycoplasmas. After the menopause the secretion is again non-acid and lactobacilli no longer predominate in the flora.

Collection of specimens

The specimen generally collected for the diagnosis of vaginitis, vaginosis or uterine sepsis is a high vaginal swab. The swab is inserted into the upper part of the vagina and rotated there before withdrawing it. So that exudate is collected from the upper as well as the lower vaginal wall. Such a Specimen however, is quite unsuitable for the diagnosis of gonorrhoea, for gonococci derived from the infected cervix tend to die off in the acid vaginal secretion and, if remaining viable, are likely to be overgrown by the vaginal commensal bacteria.

An endocervical swab must be collected for examination for gonococci. A vaginal speculum must be used to provide a clear sight of the cervix and the Swab is rubbed in and around the introitus of the cervix and withdrawn without contamination from the vaginal wall. Other swabs should also be taken from any exudate discharged from the meatus of the urethra, or a Bartholin's gland.

Swabs for Culture Should be placed in tubes of Amien's transport medium for delivery to the laboratory. If possible two swabs should be collected and submitted from

each site, the one for making films and the other for seeding cultures. Alternatively, and preferably, the physician should use a second swab to make a smear with both thick and thin areas immediately it has been collected and, after drying and heat-fixation, Submit the smear to the laboratory along with the first swab in a tube of transport medium. Smears prepared thus directly after collection of the discharge give much better results than smears prepared from swabs that have been transported to the laboratory.
For examination for trichomonas further, special specimens should be collected from the vagina and cervix, including a swab placed in clear trichomonas transport medium for microscopy and possibly culture.

Microscopic, examination

Both a wet film and a Gram_stained fillm should be examined. The wet film, between slide and coverslip, examined for the presence of motile

Trichomonas vaginalis as soon as possible after the specimen has been collected from the patient. If enough fluid cannot be expressed from the swab on to the slide, a little sterile saline may be added. If only one swab has been submitted, a sterile slide must be used. For the film must be prepared before the swab is inoculated on to culture media. The film is searched with x 10 and x 40 dry objectives for the presence of rounded or pear-shaped trichomonads showing jerky motility. The presence of polymorphs or the yeast and hyphal forms of candida should also be noted, though these are generally looked for in the Gram smear.

The Gram-stained smear should be examined particularly for evidence of candidosis (vaginal thrush), anaerobic vaginosis and, if from an endocervicaj swab, gonococcal infection. Candidosts is commonest in pregnancy and diabetes, and after the administration of broad-spectrum antibiotics, steroids or oral contraceptives. The presence of Gram-positive hyphae (pseudomycelium) in addition to numerous Gram-positive yeast forms is diagnostic. In anaerobic vaginosis there are very numerous, small Gram-negative or Gram-variable bacilli of diphtheroid morpnology (Gardnerella vaginàlis), squamous epitbelial cells covered with many such bacilli ('clue cells'), a variety of other bacterial forms, e.g. Gram-negative bacilli and vibrios, and a relative scarcity of Gram-positive lactobacilli and polymorphs. Direct tests on the foul-smelling secretion will show it contains amines and has a pH of 5 or higher.

The presence of Gram-negative diplococci intracellularly situated in a limited proportion of polymorph leucocytes, as well as some situated extracellularly, is almost diagnostic of gonorrhoea, but presumptive positive smears must be confirmed by culture, particularly in cases that may involve legal proceedings.

Culture

The specimen should be inoculated on to two plates of a rich blood agar medium, the one for incubation at 35-37°C in a humid atmosphere of air plus 5% CO2 and the other in anaerobic atmosphere with CO2. The plates should be examined after 18—24 h and again after reincubation for another 24h. As *Gardnerella vaginalis*, anaerobic cocci and bacilli, and candida may show very little growth after 18-24h . It may be helpful to have placed a 5µg metronidazole disk and a 50µg neomycin disk on the 'well' area of the anaerobic plate to assist in the recognition and isolation of anaerobes. Additional media

should be seeded when the presence of particular pathogens is expected . Culture of an additional, confluent-seeded aerobic blood agar plate bearing different antibiotic disks may assist in the separation and provisional identification of the organisms in a mixed flora.
Colonies of Candida albicans can often be recognized on the aerobic blood agar plate either by the appearance of their white colonies with spiky projections or by their growth in the inhibition zones around disks with antibacterial antibiotics. However, if the clinical features or the appearances in the Gram smear suggest that the patient may have candidiasis. it is advisable initially also to inoculate the specimen on to a plate of Sabouraud's agar or malt extract agar for aerobic incubation for 48 h at 35-37°C. The placing of a 50 unit nystatin disk and a 20µg amphotericin disk on the heavily inoculated 'well' area of the Sabouraud plate will assist in the recognition of a candida growth, distinguishing sensitive yeast colonies from resistant staphylococcal and other bacterial colonies, as well as confirming the strain's sensitivity to these therapeutic drugs in the interpretation of findings, it must be borne in mind that small numbers of candida organisms are commonly present as commensals in the healthy vagina and that the finding of a few colonies in culture is not diagnostic of candidiasis in a true infection the numbers of the organisms will often be sufficiently great for many to be seen in the stained smear.

Gardnerella vaginalis may be recognized by the growth in 48 h. of numerous very small colonies on both the aerobic and the anaerobic blood agar plate. But when the clinical features or the appearances in the Gram-stained smear suggest the patient has anaerobic vaginosis it is helpful to inoculate the specimen also on to peptone-starch-dextrose agar.
Neisseria gonorrhoeae may grow well enough on the rich moist blood agar incubated in air plus CO2, but if the clinical features or the appearances in the Gram smear suggest that there may be a gonococcal infection, the specimen should be inoculated additionally on to a plate of moist heated-blood ('chocolate') agar and a plate of the modified New York City selective medium for incubation at 35-37°C in air plus 5-10% CO2.

When uterine sepsis is suspected, the specimen should be inoculated on to a MacConkey plate and into a cooked-meat broth as well as on to aerobic and anaerobic blood agar plates, and examined for pyogenic bacteria as described above for wound swabs. When an actinomycotic infection is suspected, anaerobic culture should be continued for 7 days and the specimen should be seeded on to an additional plate of selective medium. e.g. blood agar containing metronidazole 2.5g/litre.

Non-specific genital infection (NSGK). Nonspecific (i.e. non- gonococcal) genital infection in women is generally due to Chlamydia trachomails. The chiamydial infection is primarily a cervicitis, which in over half the patients may be symptomless. it may also cause urethritis, clinical or silent salpingitis leading to infertility, pelvic inflammatory &sease or, post partum, inclusion conjunctivitis or pneumonitis in the patient's newborn baby.

Laboratory facilities for the demonstration of Chlamydia are often unavailable, and diagnosis is based on the clinical features and the laboratory exclusion of gonococcal and trichomonas infection. If facilities are available, scrapings of epithelial cells from the cervix and urethra should be collected on microscope slides for the identification of elementary Chlamydia bodies and larger inclusions in the cells' cytoplasm by immunofluorescence with specific, preferably monoclonal antiserum. Scrapings may otherwise be examined m an enzyme linked immunosorbent assay (ELJSA) test or scrapings collected in antibiotic-free transport medium for Culture in irradiated McCoy cells.

Mycoplasma infection. The role of mycoplasm in non-specific genital infections is uncertain. T strain mycoplasmas (Ureaplasma urealyzicum) have been associated with abnormalities of reproduction including infertility, habitual abortion and low birth weight but the significance of these associations is difficult to evaluate, for vaginal and urethral colonization with ureaplasma is found in a majority of sexually active women, Ureaplasmas can be cultured from cervical or vaginal swabs and the centrifuged deposit from the first 30-40ml of voided urine. Their isolation and identification is aided by their ability to hydrolyse urea.

Perform quantitative culture by making 10-fold dilutions of the specimen in small (0.9 ml) volumes of liquid medium comprising PPLO broth, horse serum, urea and phenol red indicator. Express the exudate from the swab into 0.9 ml medium to obtain a 101, dilution and prepare further dilutions up to 106. Incubate at 37°c and examine for a color change each day up to 7 days. With color changes resulting from ureaplasma growth the medium remains clear; any turbidity is a sign of bacterial contamination Which invalidates the result.

Syphilis and herpes Serologica, tests for syphilis should be performed only in cases where there is a clinical indication of this infection, but also in cases of other genital and sexually transmissible conditions, for Syphilis may be present in addition to a diagnosed gonococcal, trichomonal or chlamydial infection. If an accessible ulcer is present exudate from it should be examined by dark ground microscopy for the presence of treponemes.

Serological Diagnosis of Syphilis

Clinical Condition	Sample	Laboratory test VDRL	TPHA(treponemal antigen)	FTA
Early Syphilis				
Primary Syphilis	Serum	-or+	-or+	+usually
Secondary Syphilis	Serum	+	+	+
Latent Syphilis	Serum	+	+	+
Late Syphilis	Serum	-or +	+	+
Neurosyphilis	CSF	+	+	+
Recently treated Syphilis	Serum	-or+	+	+

TPHA; Treponema pallidum haemagglutination inhibition test
FTA; fluorescent antibody immobilization test for Treponema pallidum

In suspected genital herpes fluid from any vesicle and exudates collected from the base of ulcers should be placed in virus transport medium and submitted for Culture for herpes simplex virus .

Genital infections in men

The genital infections in men are mostly caused by the same organisms as in women, but are seldom asymptomatic In practice, urethritis is classified as gonococcal or non-gonococcal (NGU, NSU) depending on whether or not gonococci are found in a Gram film or a culture of the discharge. Most cases of NGU are caused by C. trachomatis, whilst ureaplasmas probably account for about 10% of cases. Prostatitis is rare in sexually active men and when it occurs is Usually caused by gonococci or Chlamydia. The subacute or chronic prostatitis found in older men is usually associated with the presence of coliform bacilli or enterococci. The same differences in etiology between younger and older patients apply also to epididymitis. A tuberculous epididymitis is occasionally found in patients with latent or active tuberculosis.

Balanitis and balano-prostatitis often occur as mixed infections associated with inflammation and ulceration; skin and faecal bacteria are usually present and there is often an active bacteroides component. Ulceration of the penis may be caused by herpes simplex virus, usually type 2, the Primary chancre of syphilis by Treponema pallidum the soft sore of chancroid by Haemophilus ducreyi lymphogranuloma venereum by chlamydia and granuloma inguinale by Cornybacterium granulomatis . Orchitis may be a reaction to mumps.

Collection of specimens

Urethral discharge milked from the urethra may be expressed directly On to slides for examination in Gram. stained films for gonococci. Discharge Collected in an inoculating loop should, if Possible, be inoculated immediately on to warmed plates of heated-blood agar and selective medium for the culture of gonococci . If specimens have to be transported to the laboratory for the preparation of films and inoculation into culture, as much exudate as possible should be collected on a swab and the swab at once Plunged into a tube of Amies transport medium. When prostatitis is suspected and there is no spontaneous discharge from the urethra, massage of the prostate per rectum may examination of a chancre required the careful collection of exudates and its preparation for darkground microscop. A specimen of clotted venous blood should be collected for serological examination .

Laboratory examinations

Comparable specimens are examined for pathogens on the same lines as those from infections in women. The interpretation of results is usually easier in male than female infections, for specimens of urethral discharge and exudate from ulcers are less likely to be contaminated with organisms from the perineum. Nevertheless, the distal urethra in men does possess a commensal flora, including diphtheroid bacilli, albus staphylococci , enterococci and coliform bacilli. The presence of these bacteria is unlikely to be related to infection and should be ignored.

Confidentiality. In principle, laboratory reports are confidential to the physician requesting the examination, but many laboratories dispatch batches of reports to

hospital wards and medical clinics where they are opened by secretaries, nurses or junior medical staff for distribution into the patients' records. Confidentiality is particularly important in the case of reports. whether positive or negative, on the results of examinations for sexually transmitted infections. Such reports, or at very least the positive ones, should be posted separately and directly to the physician in charge of the patient's case, and the use of code numbers rather than names merits serious consideration.

6. MENINGITIS

There is urgent need for the laboratory diagnosis of suspected meningitis, for bacterial meningitis is life-threatening and requires appropriate anti-biotic therapy at the earliest possible moment. An out-of-hours laboratory service should be available so that calls for examinations can be met at any time of the day. Preliminary findings should be telephoned at once to the physician to guide his initial choice of therapy.

The clinical signs of meningeal irritation always suggest infection of the meninges, but they sometimes occur in association with certain other acute infections not involving the meninges (meningismus) and with certain non-infective conditions such as subarachnoid haemorrhage. Infants, moreover. may have meningitis without the usual localizing signs. Laboratory examinations therefore have an important role in establishing whether or not there is meningitis as well as in determining the causal organism in cases of infective meningitis. Every patient suspected of having meningitis should have a specimen of cerebrospinal fluid (CSF) examined in the laboratory.

Most cases of meningitis fall into one of two. categories: purulent meningitis and aseptic meningitis.

In purulent meningitis the CSF is typically turbid due to the presence of large numbers of leucocytes. e.g. from 100 to several thousands per mm3, most of which are polymorphs. The majority of cases are caused by one or other of three bacteria: meningococcus, pneumococcus and Haemophilus influenzae which generally pass to the meninges from the respiratory tract via the bloodstream.

In neonates and infants, coliform bacilli. group B streptococci and, less commonly, pseudomonad, salmonellae and Listeria monocytogenes may be the cause. Infections acquired through a carelessly performed lumbar puncture, an accidental wound or an infected neurosurgicat wound may be due to pyogenic staphylococci or streptococci, coliform bacilli, anaerobic cocci or bacteroides. In patients with CSF-venous shunts, infection may be caused by Staphylococcus epidermidis or some other commensal or saprophytic bacterium. The meninges may become inflamed in reaction to an underlying brain abscess, in which case the CSF may or may not contain large numbers of polymorphs and may or may not yield a culture of the causal organism.

In aseptic meningitis the CSF is clear or only slightly turbid and contains but moderate numbers of leucocytes e.g. 10-500/mm3, most of which are lymphocytes except in the earliest stage. The great majority of cases are due to viruses (viral meningitis), particularly enteroviruses of the echo, coxsackie and polio groups. Mumps virus is a moderately common cause and a few cases are due to herpes simplex, Varicella-zoster, measles and adenoviruses. Arboviruses cause cases in countries where these viruses are common.

A few cases with CSF findings resembling those of viral meningitis are caused by leptospires (*serovars canicola* and *icterohaemor-rhagiae*), fungi (*Cryptococcus neoformans* or *Candida albicans*) and amoebae (*Naegleria* or *Hartmanella*) and underlying viral encephalitis may give a moderate lymphoeytic exudate in the CSF. It should also be noted that when antibiotic therapy is started at an early stage in a bacterial meningitis the CSF findings may be like those of aseptic meningitis

Tuberculous meningitis results from a progressive primary infection, usually Pulmonary or mesenteric, and is often associated with miliary tuberculosis It is fatal unless specific anti-tubercujous therapy is given, so its prompt diagnosi5 is essential The CSF findings often resemble those of aseptic meningitis, but the cell count is usually slightly higher, e.g. 100-50 leucocytes,rnm3 mostly lymphocytes, and a veil clot (fibrin web) often develops when the CSF is allowed to stand Undisturbed.

Collection of specimens

The Principal Specimen to be examined is of CSF collected by lumbar puncture The procedure should be attempted only by Physicians well trained in its performance and rigorous aseptic precaution of must be observed to prevent the a of infection. Only 3-5 ml of fluid should be collected, for the removal of a larger volume may lead to headache, and the rate of collection should be Slow about 4 or 5 drops a second. When there is increased intracranial pressure, the fluid may tend to spurt out, in which case the withdrawal should be quickly checked, for a large sudden removal of fluid may draw down the cerebellum info the foramen magnum and compress the medulla .

Container for CSF. The laboratory should supply fresh sterile screw-capped containers exclusively for the collection of CSF. These should not be containers that have been cleaned and sterilized after previous use for other purposes Such re-used containers may Contain bacteria from a previous specimen e.g. urine, or a culture which, although killed by the sterilization procedure may be seen in a Gram-stained film of the CSF and lead to the issuing of an erroneous preliminary report based on the findings in the film.

Lumbar puncture. The skin at this site should be disinfected with, e.g., alcoholic iodine solution. A sterile, hollow lumbar puncture needle containing an occlusive stylet should be used. The operator, wearing sterile gloves should push the needle deeply between the third and fourth lumbar spines, either in the mid line or slightly to one side of it, so that the tip of the needle, with its bevel downwards passes slightly headwards into the spinal canal, which it should reach at a depth of 4-6 cm. The stylet should then be withdrawn and. if the needle is correctly placed, the first drops of CSF should appear within a few seconds. The fluid should be allowed to fall drop by drop into the container until 3-S ml has been collected. The needle should then be withdrawn, a sterile occlusive dressing applied to the puncture site and the patient left lying down for several hours afterwards.

The specimen must be dispatched to the laboratory quickly as possible for delay may result in the death of delicate pathogens. such as meningococci, and the disintegration of leucocytes. It should not be kept in a refrigerator, which tends to kill ***H. influenzae***. If delay for a few hours is unavoidable, the specimen is best kept in an incubator at 37°C.

Blood culture. Blood for cultures should be collected at the same time as the CSF, if possible before antibiotics are given. Bacterial meningitis is often associated with a bacteriaemia and the delicate causal organism may sometimes be isolated from the blood when culture of the CSF is negative. *Septic spots* are often present on the skin in cases of meningococcal meningitis and bacteriaemia. One should be scraped and fluid expressed on to a microscope slide for Gram staining, for the cocci may be demonstrated in this material when they cannot be seen in the CSF.

Laboratory examination of CSF

The specimen should be examined with the naked eve for the presence of turbidity and any sign of contamination with blood from the puncture wound. Normal CSF is clear aid colourless like water. A yellow color may result from a previous cerebral haemorrhage. The specimen should then be examined by cell count, Gram film, culture and, if facilities are available, for its glucose and protein contents and the presence of haemophilus, meningococcal or pneumococcal antigens.

Cell count

The leucocytes in the CSF are counted by microscopical observation of well mixed, uncentrifuged fluid in a slide counting chamber. The relative numbers of polymorphs and lymphocytes should be noted, and the number of erythrocytes in specimens contaminated with blood. Normal CSF contains only 0-5 leucocytes/mm^3, mainly lymphocytes, though in neonates up to 30/mm^3, mainly polymorphs. In purulent meningitis there are usually 100-3000 leucocytes-mm^3, mostly polymorpbs, though in some cases much higher numbers. In aseptic meningitis there are usually 10-500 leucocytes/mm^3. mostly lymphocytes, though polymorphs may predominate in the earliest acute stage of the illness. In tuberculous meningitis there are usually 100-500 leucocytes/mm^3, mostly lymphocytes.

If the CSF is heavily blood stained, it is not worthwhile to attempt to make a cell Count, for numerous leucocytes derived from the blood will be present. But if there is only slight contamination with blood, the leucocytes and erythrocytes should be counted separately. The finding of leucocytes in numbers greatly in excess of 1 per 1000 erythrocytes, the approximate ratio in blood, will suggest the presence of meningitis.

When examining the wet film of CSF for the cell count, care should be taken not to mistake the rare presence of yeasts or amoebae for leucocytes. A wet film of centrifuged CSF deposit mixed with India ink will, when examined under oil-immersion, demonstrate the characteristic capsulate yeast cells of *Cryptococcus neoformans* and a wet film examined on a warm stage will show the slowly motile trophozoites of *Harimanella* or *Naegleria.*

Counting chamber. The cell count is usually performed in a modified Fuchs-Rosenthal slide. chamber, which has a *film* depth of. 0.2mm between the counting surface of the slide and the overlying coverslip. The counting surface is marked with triple lines into nine large squares, each 1mm^3 in area and subdivided into 16small squares. The volume of fluid in the film overlying five large squares is thus 1 mm^3 and the count of the cells on live large squares is thus the count per mm^3.

Dilution. CSF that is clear or only s1ightly turbid should be examined undiluted but when the specimen is highly turbid and its cell count very high, it may be necessary to dilute it 1 in 10 or I in 100 before examination. When separate counts are to be made of the leucocytes and erythrocytes, 0.85% NaCI solution should be used as diluent. If , however , the presence of large numbers of erythrocytes makes the recognition and counting of *the* leucocytes difficult, the dilution should be done with a *counting fluid* which lyses the erythrocytes and stains the nuclei of the leucocytes. A suitable fluid contains acetic acid and crystal violet, e.g.

Glacial acetic acid	1 ml
propan 1. *2* diol	2.5 ml
Crv5tal violet 1% **in** water	1.5 ml
Distilled water	100 ml

Procedure.

1. Make sure the surfaces of the counting chamber and its coverslip are clean and dry. Press the cover slip on to the support areas at the sides of the counting surface until broad bands of rainbow colors (Newton's rings) appear and indicate that close and even contact has been made.
2. Gently but thoroughly mix the diluted or undiluted uncentrifuged CSF. Take up about 0.2ml in the capillary end of a Pasteur pipette. Carefully apply the tip of the pipette to the counting surface at the edge of the coverslip and allow the fluid to run into the chamber so that is fills the whole chamber without the presence of bubbles of air and yet does not spill over to the support areas on either side. Newton's rings should still be apparent .
3. First inspect the area of the counting grid with the low power of the microscope. Defocus the condenser to make the unstained cells clearly visible. Then count the cells with a x 40 dry objective (magnification x 300 or x 400). Count the cells on five of the large squares. Include in the count any cells that overlap the innermost line of the triple-lined border on the left-hand aid distal and distal sides of the square and exclude from the count the cells that overlap the border on the night and proximal sides. Take care not to count eythrocytes as leucocytes.
4. Add together the counts for the five large squares and, in the case of diluted specimens, multiply by the dilution factor to get the count per mm^3.

Differential leucocyte count. If there is any difficulty in differentiating polymorphs and lymphocytes in the counting chamber, make a film of the cellular deposit after the specimen has been centrifuged, fix with heat, **stain** with methylene blue, Leishman or carbol thionine and examine by oil immersion to assess the relative numbers of the two types of leucocytes.

Gram film of CSF

After taking some CSF for the cell count, the remainder should be centrifuged to deposit any cells and bacteria and a film of the deposit should be stained by Gram's method. As cells and bacteria may be scanty. it is generally helpful to make a thick film within an area of about 10 mm diameter encircled by a scratch on the surface of the slide, so that the area to be searched is clearly defined. When the CSF is highly turbid and proteinaceous, part of the film should be thin, for sometimes a wholly thick film, although dried and fixed by heat, becomes washed off the slide in the course of staining.

A very careful search for bacteria should be made particularly in areas of the film where there are plenty of leucocytes, and the search should be continued for at least 10 mm before accepting the result as negative. The finding of bacterial forms resembling meningococci, pneumococci, baemophili, coliform bacilli, streptococc4 or listeriae should at once be reported to the physician, for different antibiotics are preferred for treatment of the different infections: e.g. benzylpenicillin (or chloramphenicol) for

meningococcus and pneumococcus; chloramphenicol for haemophilus: chloramphenicol, ampiciilin or cotrimoxazole for coliforms~ benzylpenicillin or chloramphenicol for group B streptococci; and ampicillin or chioramphenicol for listeria. BenzylpeniciHin and chloramphenicol are often given blind to cases of suspected meningitis before laboratory results are available, but it is important to avoid overdosage or prolonged dosage with chloramphenicol in infants, especially neonates, and the earliest indication of the type of bacteria present may be helpful in enabling the physician to change to other drugs.

When a variety of bacterial forms is seen in the film, the probability of the specimen being contaminated with live or dead bacteria should be suspected.

Culture of CSF

Immediately after centrifugation of the CSF and the removal of some of the deposit for the Gram film, the remainder of the deposit should be seeded heavily on to culture media, e.g. a plate of blood agar and a plate of heated-blood ('chocolate') agar for incubation in humid air plus 5-10% CO_2, and a tube of cooked-meat broth. Particularly when there may be a brain abscess, possibly due to bacteroides or anaerobic cocci, a further blood agar plate should be seeded for incubation for *2-5* days in an anaerobic atmosphere with 5-10% CO_2. When organisms are sufficiently numerous to be seen in the film, another blood agar plate should be seeded confluently and antibiotic disks applied, including disks with benzylpenicillin and chioramphenico!, so that sensitivity results may be obtained with minimal delay, but they must be regarded with caution, especially those for *Haemophilus influenzae.*

The cultures should be inspected after overnight incubation. Any growth should be identified and if primary sensitivity results are not available, tests with appropriate antibiotics should be done on the isolate. If no growth is apparent after overnight incubation, the plates should at once be reincubated for another day and then again inspected for growth. if the plate cultures remain free from growth and turbidity develops in the cooked-meat broth, the broth should be filmed and subcultured on to blood agar and heated-blood agar plates. incubated aerobically and anaerobically.

Biochemical tests

The supernatant from the centrifuged CSF should be tested for its content of glucosc and protein. Normal CSF contains 2.2-4 mmol glucose/litre (about 60% of the plasma glucose value) and *0.15-0.4* g protein/litre (in neonates up to 1.5 g protein/litre). In purulent bacterial meningitis the glucose concentration is reduced (0-2 mmol/litre) and the protein concentration increased (0.5-3 g/litre). in aseptic (viral) meningitis the glucose concentration is normal and the protein concentration raised a little **(0.5-1** g/litre).

Bacterial antigens

If appropriate reagents are available, a rapid indication of the type of infection may be obtained by the performance of a coagglutination or counter-immunoelectrophoresis test on the CSF (or blood serum) to demonstrate the presence of the antigens of meningococci of serotype A, B or C. or the capsular antigens of the commoner types of

pneumococci or Pittman's type b of *Hoemophilus. Influenzae.* Convenient kits for the coagglutination test are available commercially.

Viral meningitis

If the clinical findings and the results of microscopically and biochemical examinations of the CSF suggest the presence of aseptic meningitis, an attempt may be made to isolate the virus from the CSF. a throat swab or a specimen of faeces, and paired sera may be examined for viral antibodies. Some CSF should be kept at -‘70°C until it can be inoculated into cell cultures, which *may* yield an echo, Coxsackie's or herpes virus. A throat swab submitted in viral transport medium may be cultured for these and mumps virus, *and* faeces may be cultured for echo. Coxsackie's and polio virus.

Tuberculous meningitis

When a tuberculosis infection is suspected. the centrifuged deposit of the CSF should be examined in a Ziehl-Neelsen stained film for acid-a bacilli and cultured on one or two slopes of Löwenstein4enscn medium. The film should be searched for at least *5* mm before being accepted as negative. As in purulent meningitis, the glucose content of the CSF is reduced and the protein, content increased.

Leptospiral meningitis

When the CSF appearances are those of aseptic meningitis and the clinical features. e.g. conjunctivitis, jaundice or nephritis. suggest that the infection may be leptospiral, paired sera should be collected to demonstrate a rising titre of leptospiral antibodies in the serovar-specific microscopical agglutination test or the genus. specific complement-fixation and sensitized erythrocyte tests. Occasionally, motile leptospires may be seen in the CSF under darkground illumination and occasionally leptospires may be cultured from the CSF or blood inoculatcd into Korthoff's or other leptospiral medium.

7.GASTROINTESTINAL INFECTIONS

The commonest specimens examined for gastrointestinal infections are those of faeces horn patients with diarrhoea, with or without abdominal pain or vomiting. Formed stools may be submitted from patients suspected of having *enteric* fever, helminthiasis or the subclinical carriage of an intestinal pathogen, and clotted blood may be submitted for serological examination for suspected enteric fever or an intestinal virus infection. The common infectious causes of diarrhoea in adults and children over *2-3* years old are infections with *Campvlobaczer* spp., *Salmonella typhimurium* and other animal-derived salmonellas and *Shigella sonnei*, and food-poisoning due to these and other bacteria, e.g. *Staphylococcus aureus*, *Clostridium perfringens (C. welchi)*, *Bacillus cereus* and *Vibrio parahaemolyticus*. A moderate number of cases are caused by the protozoon, *Giardia intestinalis (G. lamblia)* and the fungus. *Cyptosporidiurn*, and a few cases by *shigella flexneri, Salmonella typhi, Salmonella paratyphi B, Aeromonas hydrophila, Plesiomonas shigelloides* and *Yersinia enterocolitica.*

In infants under 3 years old, many cases of gastroenteritis are caused by rotaviruses and certain other viruses, and by intestinal pathogenic strains of *Escherichia coli*. These organisms also cause some infections in adults. e.g. in persons travelling abroad and encountering an enterotoxigenic strain of *E. coli* of a serotype not previously encountered at home ('traveller's diarrhoea'). Particularly in childhood, diarrhoea may be caused by infections elsewhere than in the gastrointestinal tract. e.g. by respiratory, urinary and septicaemia infections, and by certain non-infective conditions such as thc food allergies

Various exotic intestinal pathogens may be acquired by persons travelling abroad and cause diarrhoeal illness soon after the traveller has returned. These pathogens include *Vibrio cholerae* , *Shigella dysenteriae*. *Shigella boydi, Salmonella paratvphi A* and *Entamoeba histolytica*. Physicians should be advised always to inform the laboratory about any recent foreign travel by the patient so that the microbiologist is warned to perform the special examinations required for demonstration of the exotic pathogens.

In patients treated with antibiotics, e.g. for prophylaxis during intestinal surgery, severe enterocolitis may be caused by a drug-resistant strain of *Staphylococcus aureus* and a life-threatening pseudomembranous colitis by *Clostridium difficile*. Milder, simple diarrhoea often follows prolonged treatment with any of a variety of antibiotics which deranges the bowel flora and predisposes to superinfection with various drug-resistant bacteria, *Candida albicans* or *Crvptosporidium.*

Collection of *specimens*

Whenever possible, a specimen *of faeces* should be obtained. A *rectal swab* is unsatisfactory unless it is heavily charged and visibly stained with stool collected from the rectum, not anus. A swab heavily charged with faeces wiped from toilet paper is usually satisfactory. If enteric fever is suspected, a specimen of venous blood should be collected for *blood culture*. If food-poisoning is suspected because a cluster of cases are related to the eating of a common foodstuff, *a* **sample** of the suspect food should be collected. For serological examinations, paired acute and convalescent samples of

clotted blood should be collected at an interval of about 10 days in suspected enteric fever and 2-4 weeks in suspected viral infection.

Stool. The specimen may be collected from stool passed into a clean bedpan, unmixed with urine or disinfectant, or from the surface of heavily soiled toilet paper. The container is a 25 ml screw-capped. wide-mouthed glass or plastic bottle with a 'spoon' projecting from the underside of the cap. Collect 1-2 ml of stool on the spoon and insert it, carried on the spoon. into the bottle. Take care not to soil the rim or outside of the bottle. Apply the cap tightly. Do not collect several spoonfuls or attempt to fill the container. Transmit the specimen quickly to the laboratory. If delay is unavoidable, and particularly when the weather is warm, collect the stool in a container holding about 6 ml buffered glycerol saline transport medium.

Examination of Stool

The sample should first be inspected with the naked eye for its consistency, whether formed or fluid, the presence of mucus, pus and blood, indicative of severe dysentery, and the presence of helminths.

It is usual then to proceed to culture on media suitable for isolation of the common bacterial pathogens. It is generally sufficient to culture for campylobacters, salmonellas and shigellas, and to search for other pathogens only when the clinical features or epidemiological circumstances suggest the need to do so, e.g. in infantile or post-antibiotic diarrhea, in suspected outbreaks of food-poisoning, when the patient has recently returned from travel abroad, and when there has been failure to isolate a common pathogen in a case of persistent diarrhea.

Some bacteriologists, for instance, search for Clostridium difficile in all hospital patients. others for cryptosporidium in all young children and immunodeficient patients.

In well staffed laboratories it is recommended that all specimens should be examined microscopically for pus and red cells, protozoa. Protozoal and cryptosporidial cysts. helminths and helmjnthic ova.

A suspension should be prepared for inoculation into the different media. Unless the sample is fluid a portion of it should be suspended to give a I in 10 dilution in 2-3 ml of phosphate-buffered (pH 7.3) saline or 0.1% peptone water.

Campylobacter. Inoculate one or two loopfuls of the suspension on a plate of campylobacter selective medium. e.g. the Skirrow or Preston medium and incubate for 48 h at 42—43°C under microaerophilic conditions (5-6% O_2, 7-10% CO_2 in H_2 or N_2,). If the stool is more than 24 h old; also inoculate one or two loopfuls into a tube of Preston campylobacter enrichment broth incubate for 24 h at 42-43°C, and then subculture on to a plate of campylobacter selective medium.

Salmonella and Shigella. There is a wide choice of selective and enrichment media for these organisms. A good procedure for routine examinations is to seed a plate of one selective medium, e.g. deoxycholate citrate agar (DCA), and a tube of one enrichment broth e.g. selenite F broth. lnoculate one or two loopfuls of the faecal suspcnsion on to the DCA plate, stroking out with care to yield many separate colonies. and one or two drops of the suspension into the selenite F broth, and incubate aerobically for 18-24 h at 37°C.

Inspect the plate for pale (non Lactose-fermenting) enterobacterial colonies, pick and prepare a pure subculture (e.g. on a nutrient agar or urea agar slope) from each of any different morphological types of pale colonies, and identify the subcultures by biochemical reactions and serological study. Streak out a loopful of the selenite culture on DCA, incubate this plate overnight and examine for pale colonies as before. The stool may contain non-lactose-fermenting commensal bacteria as well as a non-lactose-fermenting pathogen and it is therefore inadvisable to conclude that pathogens are absent from the examination of only a single pale colony. If the only pale colonies are crowded and touching other colonies, pick one as cleanly as possible and plate it out on MacConkey agar to obtain pure. Well separated pale colonies the following day. Do not base the identification of a salmonella or shigella biochemical or serological tests made on a possibly mixed culture.

Xylose lysine deoxycholate (XLD) agar has considerable advantages as a primary plating medium for faeces and its *use* instead of DCA *Is* recommended It is less inhibitory than DCA to S.*dysen:errae* and S. *flexneri.* and it distinguishes most saimonellas (red colonies with black centres) from shigellas (red colonies without black centres) and most non-pathogenic Conforms (yellow colonies). The chances of isolating a scanty salmonella or shigella will be increased if additional selective and enrichment media are seeded. There are advantages in seeding a MacConkey agar plate and, for the isolation of salmonellas, a plate of brilliant green MacConkey agar, a plate of Wilson & Blair's brilliant green bismuth sulphite *agar* (BBSA) and a tube of tetrathionate broth

Other food-poisoning bacteria

If the epidemiological circumstances suggest that the patient has been involved in an outbreak of food-poisoning and if the sample of his faeces has been collected within 3 days of the start of his illness, examine the sample for *Clostridium perfringens, Staphylococcus aureus, Bacillus cereus* and *Vibrio parahaemolyticus* as well as for campylobacter salmonella and shigella. If possible also obtain a sample of the suspected food-stuff for culture.

Clostridjum perfringens. Food-poisoning may be caused by either heat-sensitive strains (spores killed *in* 10 min at 80°C) or heat-resistant strains (spores survive for 10 mm at 80-100°c) *of* this organism, though the resistant strains are more likely to be the cause of outbreaks due to well cooked foodstuffs as their spores are more likely to survive the cooking. The mere finding of the organism in the stool if it is treat-resistant, does not indicate that it has a causal role in the illness, for normal subjects commonly have 1000-10 000 bacilli or spores of *C. perfringens* per gram of faeces. Culture should therefore be done by a semi-quantitative method, for faeces collected from patients at the height of the illness commonly contain 1 000 000 or more *C. perfringens* per gram and it is only the finding of such high counts that should be reported as probably significant.

The demonstration that strains isolated in large numbers from several patients and the suspected food are of the same serotype provides stronger evidence of their causal role.

It may also be helpful to examine the specimens of faeces for C. perfringens enterotoxin, for the finding of this toxin in significant amounts may confirm the

diatmosis in an outbreak when the cultural results are equivocal. The examination for toxin may be made in a reference laboratory on faeces transmitted by post, but reversed passive latex agglutination kits are now available commercially (e.g. Oxoid) for testing in the general clinical laboratory.

Staphylococcus aureus. Plate out a few loopfuls of a 1 in 10 saline suspension of the faeces on plates of blood agar, MacConkey agar and a selective medium. e.g. 6% NaCI nutrient agar. mannitol salt agar or phenoiphthalein phosphate agar plus 1250 units polymyxin B/litre. Incubate aerobically for 18-24 h at 37°C and examine for colonies of S. aureus. In an outbreak, send subcultures from each patient to a reference laboratory for phage typing and tests for enterotoxin production.

S. aureus may often be isolated from the stool of healthy persons, so that its isolation from the stool of a patient with diarrhea is not proof of a causal role. Identity of phage-type among the isolates from several patients in an outbreak and from a specimen of suspected foodstuff is fair evidence that the outbreak is due to staphylococcal food-poisoning. Some outbreaks, however, arc caused by a foodstuff that has been heated, e.g. pasteurized, at a temperature sufficient to kill the staphylococci though insufficient to inactivate the more thermostable enterotoxin. In such cases the diagnosis requires the demonstration of staphylococcal enterotoxin in the faeces or food. Kits for the detection of detection of staphylococcal enterotoxins A. B, C and D by reversed passive latex agglutination are available commercially (Oxoid).

Bacillus cereus. Particularly when the outbreak is attributed to the eating of a rice dish, inoculate loopfuls of faecal suspension on to plates of blood agar, MacConkcy agar and, if available, the selective medium of Holbrook & Anderson. After aerobic incubation for 18-24 h at 37°C, look for large rough pale colonies on MacConkey agar and blue colonies surrounded by a precipitate on Holbrook & Anderson's medium. Identify them by their appearance in a Gram film and other tests

Vibrio parahaemolyticus

1. Particularly in outbreaks attributed to raw sea fish and shellfish, plate a few loopfuls of a 1 in 10 suspension of faeces on thiosulphate citrate bile sucrose (TCBS) agar and incubate aerobically for 18-24 h at 37°C.
2. Also inoculate a portion of the faecal suspension into an equal volume of double strength alkaline (pH 8.8) peptone water, incubate 18-24 h at 25°C, then subculture on to a TCBS plate and incubate for 18-24 h at 37°C.
3. inspect the plates for large (2-5 mm) blue or green (non-sucrose-fermenting) colonies. Demonstrate they contain vibrios by Gram filming and that they are oxidase positive.
4. Suspend a colony in 3% NaC1 solution and inoculate drops of the suspension into media for identifying V. parahaemolyticus, which is motile, ferments glucose but not sucrose with the production of acid but not gas, grows in alkaline peptone water with 8% NaCl but not in peptone water without NaCI or on CLED medium, and fails to agglutinate with vibrio cholerae O1 antiserum. Send a subculture to a reference laboratory for scrotyping.

Infantile gastroenteritis

Most cases appear to be caused by particularly rotaviruses. which only rarely cause gastroenteritis in children more than a few years old or in adults. As yet. tests are general not done to demonstrate viruses in the samples of stool and a viral etiology is assumed from the failure to demonstrate a bacterial pathogen. When facilities are available a centrifuged concentrate of the stool may be examined by the electron microscope, which in the acute phase of the illness will show the presence of large numbers of rotaviruses.

Rotavirus antigen may also be demonstrated in the stool by a serological method (e.g. ELISA or latex agglutination), but the use of such tests requires careful and informed laboratory monitoring.

Enteropathogenic Escherichia coli. These enteropathogenic strains of E. coli often cause gastroenteritis in neonates and infants under 3 years old and occasionally also in older subjects. Strains of certain serotypes of the species possess the intestinal pathogenic properties more commonly than strains of other serotypes. Thus the demonstration that a dominant strain in a patient's stool belongs to one of the former serotypes suggests that it has a pathogenic role, but some strains of these serotypes lack the pathogenic properties. Thus, reliance should not be placed on serotyping as indicative of enteropathogenicity, otherwise E. coli may wrongly be judged the cause of a diarrhoeal illness that is due to some other, undetected, e.g. viral, cause.

There is still no test for enteropathogenic properties suitable for routine application to faecal isolates. It is therefore probably wisest not to examine faeces for *E. coli* in sporadic cases of infantile diarrhoea, but only to do so when *there* is an outbreak among infants and other common intestinal pathogens have not been found.

1. Plate a loopful of faecal suspension on a blood agar plate and a MacConkey plate and incubate 18-24 h at 37°C.
2. Test from 3-10 *E. coil-like* colonies from the blood agar plate for slide agglutination within 1 min with pools of polyvalent sera for enteropathogenic serotypes of *E.* ***coil***.
3. If any colony, gives a strong reaction with one of the pools. Inoculate the remainder of it on to a nutrient agar slope and incubate the slope overnight.
4. prepare a dense suspension from the slope culture in saline and test it by slide agglutination with monovalent sera to identify the K antigen.
5. Then heat the suspension for 1 h at 100°C. cool it and repeat the slide tests with the monovalent sera to identify the O antigen. Confirm the O serotype by demonstrating tube agglutination to the titre of the serum.

Enterotoxigenic Escherichia coil. Strains of E.coli producing heat-stable or heat-labile enterotoxin may cause diarrhea in adults and children who have not previously encountered them and should be sought particularly in cases of 'traveller's diarrhoea'. They may be cultured from stool on MacConkey and blood agar media and serotyped. Not all strains of commonly toxigenic serotypes produce enterotoxins and cultures may be examined for toxin production in a reference laboratory or for heat-labile enterotoxin with a reversed passive latex agglutination kit (Oxoid). Most commonly known strain is verocytotoin producing E. coli it is responsible for haemolytic uraemic syndrome. It is

characterized by that it is sorbitol negative so it can be isolated on sorbitol MacConkey agar and gives pale yellow colonies.

Microscopy for protozoa, cysts and ova

Usually a stool sample is not examined microscopically unless the clinical particulars or failure to demonstrate an alternative pathogen suggests that the patient's illness may be due to amoebiasis, giardiasis. balantidiosis, cryptosporidiosis or helminthiasis, but if sufficient staff is available, all specimens should be examined microscopically. A wet film of a concentrate of the stool should be examined for protozoa, protozoal cysts and helminth ova, and a stained film for the oocysts of cryptosporidium. An adhesive, Sellotape-tipped swab should be applied to the perianal skin and examined microscopically for threadworm (enterobius) ova.

Vegetative amoebas and giardias. if possible, obtain a fresh, warm specimen of stool. Select a portion, preferably with mucus or pus, and emulsify it in saline solution or saline containing 0.5% eosin on a slide warmed to 37°C. Apply a coverslip and at once examine the film with a x 40 dry objective for unstained, motile trophozoites, pus cells and erythrocytes.

Cysts and ova. Microscopic examination for protozoal cysts and helminthic ova should be done on a film of a concentrate of the stool (e.g. x 20).

1. Pipette 8 ml of 10% formol (4% formaldehyde) saline into a 28 ml Universal container. Thoroughly emulsify about 1 g faeces in the solution by shaking or by rubbing with a swab stick.
2. Add 2 ml diethyl ether to the suspension, shake vigorously for I mm, then centrifuge at 650 *g* (e.g. at *c.* 1000 rev/min) for exactly 2 mm.
3. With a swab stick loosen the debris floating near the surface of the ether. Then discard the debris with the ether and supernatant saline.
4. Withdraw a drop of the centrifuged deposit with a Pasteur pipette, place it on a microscope slide, add a drop of 2% iodine in 4% potassium iodide solution, apply a coverslip and seal the film with Vaseline.
5. Search the film thoroughly with a x 40 dry objective for the cysts of entamoeba and giardia and the ova of helminths such as ascaris, schistosoma, trichinella and trichuris. Check the morphology of any such bodies with the x 100 oil-immersion objective.

Identify the bodies seen by comparison with illustrations of their morphology and estimation of their size. As size is a helpful identifying feature, use a graduated eye-piece micrometer to measure the diameter of the suspect forms.

Giardiasis. Excretion of the giardia trophozoites and cysts is often intermittent, so microscopical examinations should be made on several separate collections of faeces. A rapid enzyme-linked immunoassay (ELJSA) has been described, which is more Sensitive and less laborious than microscopical examination

Cryptosporidiosis. When other pathogens have not been found, or in children, travellers or immunodeficient subjects with diarrhoea, examine fresh faeces for the oocysts of cryptosporidium . These newly spherical structures , usually 4-5 µm in diameter, resemble weakly acid-fast organisms in being moderately resistant to both the entry and exit of stains, and this property is exploited to facilitate their recognition in

films. A quick and easy method of demonstration is by fluorescence microscopy of a film stained with auramine and counterstained with carbol fuchsin

1. Make a thick smear of undiluted faeces with some thin areas. Dry it in air, fix it in absolute methanol for 3 mm and again dry it in air.
2. Immerse the fixed smear in auramine-phenol (0.03 g auramine-0. 3 g phenol. 97 ml distilled water. or as supplied by Infrakem) for 5 mm at room temperature. Rinse with tap water.
3. Counterstain for 10 seconds in strong Ziehil-Neelsen carbol fuchsin at room temperature. Rinse in tap water and dry in air.
4. View the unmounted film under an incident light fluorescence microscope with a dry objective at a magnification of x 109 or **x** 200 as for mycobacterium fluorescence microscopy. Oocysts appear as bright yellow-fluorescing spheres with dark centers on a dull red background. Yeasts do not fluoresce. Re-examine positive smears at x 1000 by oil-immersion.

If a fluorescence microscope is not available, a modified Ziehil-Neelsen stain or, Dreferably, the safranmne-methylene blue stain may be used. The latter shows orange ring-shaped oocysts on a blue-stained background.

Microscopy for staphylococcal enterocolitis

When this life-threatening condition is suspected, e.g. in a patient with severe diarrhea following intestinal surgery and antibiotic treatment, a bacteriological diagnosis is urgently required. Make a Gram-stained film of the stool and examine it for very numerous Gram-positive cocci largely replacing the normal mixed bacterial flora. At once telephone the finding to the physician. Then culture the stool as described for staphylococcal food-poisoning (see above) and determine the antibiotic sensitivities of any isolate.

Other intestinal pathogens

Culture of stool for other intestinal pathogens should be attempted when the circumstances suggest they may be present.

Clostridium difficile. Always examine for this organism in cases in which pseudomembranous colitis is suspected. e.g. where there is severe, often blood-stained diarrhea and toxaemia in a patient who has been receiving antibiotics especially clindamycin or lincomycin. The condition poses an immediate danger to life and treatment with an effective antibiotic. e.g. oral. vancomycin, is urgently required. The clostridium is also present in many cases of milder antibiotic-associated diarrhea and should be sought when other pathogens are absent .

Vibrio cholera. This important pathogen is not indigenous in most countries, but its presence should be sought in patients suffering from diarrhea just after returning by air from a country in which cholerae is known to be present.

1. Culture the stool in alkaline peptone water, on TCBS agar and on tellurite tauro. cholate gelatin agar.
2. Identify yellow (sucrose-fermenting) colonies on TCBS agar as V. cholera by demonstrating the organisms are motile Gram-negative vibrios, oxidase positive and capable of growth on CLED and other salt-free media.

3. Identify a classical cholera strain of V. cholera by demonstrating that it reacts with V. cholera O1 antiserum in a slide agglutination test.
4. Recognize non-Ol (non-cholera) strains of V. cholera by their failure to react with the O1 antiserum while giving slide agglutination with V. cholerae H antiserum.
5. Send a subculture to a reference laboratory for confirmation of an O1 strain and O serotyping of a non-Ol strain.

Aeromonas hydrophila. This organism can cause severe gastroenteritis, though in most areas the number of reported cases is small. It may grow on MacConkey, deoxycholate and other enteric agars, some strains fermenting lactose and others not, but if it is to be sought a selective agar such as sheep blood agar with 15 mg ampicillin/litre should be used. Incubate cultures on this last medium for 24-48 hat 37°C, flood the plate with 1% sodium dimethyl-p-phenylenediamine mono-hydrochloride (oxidase reagent) and recognize the aeromonas colonies by their purple-black color and surrounding narrow zone of haemolysis. Pick quickly to subculture for identifying biochemical tests .

Plesiomonas shigelloides. This organism causes a few cases of diarrhea in many countries. It may be detected by its growth on MacConkey and DCA media, usually as pale, but sometimes pink colonies, which may be distinguished from those of shigella and escherichia by their oxidase-positive reaction.

Yersinia enterocolitica. This organism sometimes causes simple gastroenteritis. Also examine stool for it in cases of suspected mesenteric lymphadenitis and consider relevant serological investigation. It may sometimes be isolated in blood cultures incubated at 22-25°C.

1. Inoculate several drops of a I in 10 suspension of the stool in buffered saline into a tube of selenite F broth or phosphate-buffered saline, pH 7.6 and hold the culture for up to 6 weeks at 4°C.
2. Inoculate a loopful of the stool suspension, and at weekly intervals thereafter loopfuls of the selenite broth culture, on to pates of DCA or CIN medium (Yersinia Setectiv agal Base, Oxoid CM 653. plus antibiotic supplement, Oxoid SR1O9) and incubate for 24 h at 32°C.
3. Inspect the DCA plates for pale (non-lactose-fermenting) colonies and the Yersinia selective agar plates for colonies with dark red centres. Identify the organism as *Y. enserocolirica* by demonstrating it is motile at 22°C. ferments sucrose at 22°C, hydrolyses urea at 35°C. It may also be identified by the API 20E kit.

Reporting

Consideration should be given as to whether antibiotic sensitivities should be reported to the physician along with the finding of a particular pathogen. In simple diarrhoea due to *Salmonella typhimurium* or other animal-derived salmonella, *Shigella sonnei, Shigella flexneri* or enteropathogenic *Escherichia coil,* treatment with antibiotics is rarely beneficial and may be harmful in prolonging the illness and the duration of carriage of the pathogen.

A report of the finding of such a pathogen with its sensitivities may suggest to an ill-informed physician that he should prescribe antibiotic treatment. A note of the

sensitivity results should of course be kept in thee laboratory and if the physician enquires about them, the circumstances in which antibiotic therapy may be beneficial rather than harmful may be discussed with him. Antibiotic treatment will be positively required for enteric fever and may be indicated when there is evidence of invasiveness (e.g. septicaemia) with other gut pathogens, or severe dysentery.

Whilst a diagnosis of enterocolitis due to *Clostridium difficile* is a signal to stop any current antibiotic therapy and will demand urgent treatment with, e.g. oral vancomycin, the significance of finding this organism in cases of simple diarrhea in persons who have not been treated with antibiotics is unclear. *C. difficile* is found in the stool of many healthy infants and is unlikely to be the cause of diarrhea in children under 2 years old. Such cases should not be treated with antibiotics.

Negative findings should be reported in terms only of the organisms that were sought and not found. e.g. ‘No campylobacter, salmonella or shigella isolated’. and not in general terms. e.g. 'No pathogens found’, for the latter type of report may suggest that examinations were made for all possible kinds of pathogens. including viruses, protozoa. fungi and helminths.

Any isolation of an infectious enteric pathogen should be notified at once to the local public health authority to prompt the investigation of outbreaks and the institution of preventive measures.

8. URINARY TRACT INFECTIONS

Samples of urine from patients with suspected infections of the urinary tract are the most numerous, e.g. 30-40%. of the different kinds of specimens received in most clinical laboratories. The schedule for their routine examination should therefore be carefully determined with a view to obtaining the necessary diagnostic information with the greatest possible economy of labor and resources.

The examinations generally made are the microscopical examination of a wet film of uncentrifuged urine to determine whether polymorphs ('pus cells') are present in numbers indicative of infection in the urinary tract, and the semi-quantitative culture of the urine to determine whether it contains a potentially pathogenic bacterium in numbers sufficient to identify it as the causal infecting organism ('significant bacteriuria').

The chemotherapy of a proven infection may be guided by in-vitro sensitivity tests on the pathogen isolated in culture and the outcome of therapy assessed by examination of the urine at the conclusion of treatment. Follow-up examination of patients who have had urinary tract infection is advisable because a relapse may be clinically silent.

The common symptoms of urinary tract infection are urgency and frequency of micturition, with associated discomfort or pain. The commonest condition is cystitis, due to infection of the bladder with a uropathogenic bacterium, which most frequently is Escherichia coli but sometimes Staphylococcus saprophyticus or, especially in hospital-acquired infections. *Klebsiella pneumoniae var. aerogenes or oxyloca, Proteus mirabilis,* other coliforms and *Pseudomonas aeruginosa or Streptococcus faecalis.* Candida infection may occur in diabetic and immuno-compromised patients. Rarer infecting organisms include *Streptococcus agulacziae, Streptococcus milleri,* and other *streptococci,* anaerobic *streptococci* and *Gardenella vaginalis.* There has been much debate on the significance of so-called fastidious organisms in urinary tract infection.

More serious bacterial infections are acute pyelitis and pyelonephritis in which the symptoms usually include loin pain and fever and which may be accompanied by a bacteriaemia detectable by blood culture. The causative organism may be any of those that cause cystitis, but *Staphylococcus aureus* is responsible for some of the cases.

Patients with signs or symptoms of urinary tract infection sometimes produce samples of urine that show pus cells but do not yield a significant growth of bacteria on routine culture.

The explanation may be that the patient has been taking antibiotics prescribed on a previous, occasion. Alternatively, he may have an infection with an organism that does not grow' on the media normally used for routine investigations. In such cases it is important to consider genitourinary tuberculosis or gonococcal infection and infection with nutritionally exacting or anaerobic bacteria. But many patients with frequency and dysuria do not have a bacterial infection of the bladder, nor significant numbers of bacteria in their urine (abacterial pyuria). Their condition is known as non-bacterial urethritis or cystitis, or the urethral syndrome. The cause of which may be urethral or bladder infection with a chlamydia, ureaplasma, trichomonas or virus, which often remains unrecognized.

Screening out negatives

About 70-80% of the urine specimens received in a clinical laboratory are found on full microscopical and cultural examinations to be free from evidence of infection in the urinary tract. A variety of chemical and automated methods have been tried for the detection of the negative specimens, but none has yet been generally accepted as sufficiently reliable for its purpose. Recently. it has been reported that the finding of negative results in all of three chemical tests for nitrite, blood and protein, performed by a rapid automated dip-strip method (N-Labstix. Ames), predicts the absence of bacteriuria in *about* half of the culture-negative specimens. which may then be discarded.

Significant bacteriuria

The specimen most easily and therefore most commonly collected is mid-stream urine (MSU).

Although the greater part of the urinary tract is devoid of a commensal flora and bladder urine ii an uninfected person is free from bacteria, a specimen of spontaneously voided urine is to be contaminated with some commensal bacteria from the urethral orifice and perineum, particularly in females, even when the most careful precautions are taken to prevent such contamination. As these contaminating commensals include the very bacteria, such as E. coli and S. saprophyticus, which are the commonest organisms to infect the urinary tract, the simple demonstration that bacteria of one of these species are present in the sample of urine is not poof that it has been derived from an infection in the urinary tract.

Proof of a urinary tract infection requires the demonstration that the potential pathogen is present freshly voided urine in numbers greater than those likely to result from contamination from the urethral meatus and its environs . The observations suggested that this number, taken to indicate significant bacteriuria , is about 100 000/ml. In true infections, in the absence or chemo therapy , the number of the infecting bacteria is likely to be at least as great as this. Accordingly a quantitative method of culture is adopted to estimate the number of viable bacteria in the specimen.

When properly collected specimens of urine are examined, contamination accounts for less than 10^4 organisms/ml and usually for less than 10^3/ml. Counts due to contamination are variable and the colonies often of diverse species. Specimens from urinary tract infections almost always contain more than 10^4 organisms/ml, usually more than 10^5/ml and often up to 10^8/ml. These high counts, which are fairly constant in serial specimens from the same patient, reflect bacterial multiplication in the urine in vivo and are accepted as indicating significant bacteriuria. The growth obtained in such cases usually represents a single infecting species, though some infections with two species. e.g. *E. colil* and *S. faecalis*, are encountered.

Significant bacieriuria (count $>10^5$/ml in a carefully taken and promptly examined sample) may sometimes occur in the absence of symptoms and pyuria in patients who subsequently develop symptoms of urinary tract infection. e.g. in pregnancy. The detection of such asymptomatic bacteriuria is of value for there is good evidence of its association with the development of pyelonephritis in some patients.

When, the specimen has been collected from the bladder by suprapubic aspiration or a freshly inserted urethral catheter, the absence of contamination may be assumed and the presence of even small numbers of bacteria must be regarded as significant.

Specimen collection

Specimens of urine are generally collected in plastic or glass Universal containers, but midstream specimens from females are more conveniently collected in a wide-mouthed container such as a 12 oz *(350* ml) honey-pot or a sterile waxed cardboard container.

From male patients, a mid-stream specimen of urine (MSU. the middle of the urine flow) is collected. From females, in whom it is more difficult to avoid contamination with organisms from the ano-genital region, a catheter specimen of urine (CSU) was commonly collected in the past, but catheterization for this purpose is no longer considered justifiable because it carries a 2-6% risk of introducing and initiating infection. A CSU is nowadays taken only if there are special indications for its requirement or in the course of a cystoscopic investigation. Routinely, therefore, MSU samples are now submitted from women and when carefully taken these compare favorably with catheter specimens. The female patient passes urine with the labia separated and the middle of the stream is collected for examination.

The collection of a clean specimen of urine from children and young infants poses problems. Collection in a bag held with adhesive tape over the genitalia inevitably yields a contaminated, and generally a heavily contaminated specimen. However, a 'negative' finding, when occasionally obtained, may be of value. Otherwise, urine may be aspirated from the bladder into a syringe with a needle introduced aseptically through the skin and abdominal wall just above the pubis (suprapubic stab).

If tuberculosis of the urinary tract is suspected, the first urine passed in the day (early morning urine) is the most suitable specimen. Three complete early morning urines should be sent to the laboratory, where they are centrifuged and. their deposits examined by microscopically and cultural tests. The individual specimen should be refrigerated pending processing.

In the investigation of urethritis and prostatitis, the initial flow of urine, rather than a midstream specimen should be examined.

Transport of specimen. Once collected, a specimen of urine must be transported to the laboratory without delay. For urine is an excellent culture medium and contaminating bacteria, can readily multiply to reach apparently significant numbers. If a delay of more than 1-2 h is unavoidable, the multiplication of bacteria in the urine should be prevented by storage in a refrigerator at 4°C, or by transport in some form of refrigerated container, or by collection and transport in a container with boric acid at a final, bacteriostatic concentration of 1.8%. When samples of urine not so treated are delayed more than 5 h in transit to the laboratory, the doctor should be informed and the samples discarded, for positive findings may be misleading. Collection and transport on a *dip-slide* avoids the difficulty of bacterial multiplication before quantitative culture, but does not provide for microscopical examination of the cellular content of the urine.

Microscopy of urine

Microscopical examination of urine is done principally to detect the presence of increased numbers of polymorphs (pyuria) as an indication of infection in the urinary tract when culture may fail to show significant bacteriuria, either because bacteria are being killed as a result of antibiotic therapy or because the infecting organism is one that is unable to grow on the routinely used media. e.g. the tubercle bacillus, a nutritionally exacting or anaerobic bacterium, a chlamydia or a ureaplasma. Such a finding of sterile or abacterial pyuria serves as an indication that further, special methods of examination should be used to detect the pathogen.

Microscopical examination is laborious, however, and may yield misleading results if performed cursorily by insufficiently experienced staff. They reserve it as a special investigation in selected cases, as when examining repeat specimens from patients with urinary tract symptoms persisting after the failure of an initial culture to demonstrate significant bacteriuria, in suspected pyelonephritis or renal tuberculosis, and in cases where information is urgently required to differentiate a urinary tract infection from appendicitis or some other abdominal condition requiring immediate surgery. It is not unreasonable to omit microscopy and rely on culture for examination of the very numerous specimens of urine submitted from general practice or out-patient clinics from patients with symptoms of simple cystitis or urethral syndrome and those undergoing routine or geriatric or ante-natal assessment. If this is the policy, the clinician must ensure that the specimen is properly taken before antimicrobial treatment and submit information about any previous therapy.

In the past, the microscopical examination was commonly done on a wet film or Gram-stained film of deposit centrifuged from the urine, as the concentration by centrifugation made it easier to detect scanty bacteria or cells. Nowadays centrifugation is not recommended, for the presence Of scanty bacteria or polymorphs is unlikely to be significant; the procedure is laborious, and Unless it is done in such a way as to give a standard degree of concentration, e.g. x 10 or x 20, a sufficiently reliable estimate of the number of Polymorphs cannot be made.

Some polymorphs are usually present in the urine of healthy, uninfected persons, and it is only if their number is clearly greater than the normal values that the finding of 'pus cells' is indicative of urinary tract infection. Generally it is accepted that the leucocytes should be found in numbers at least as great as 10^4/ml before the presence of pyuria is suggested. Thus the microscopical examination must be done in such a way as to provide a reliable estimate of the leucocyte numbers.

Wet film examination. A leucocyte count sufficiently accurate for general purposes may be obtained from examination of a wet film of uncentrifuged urine, provided that the area of the microscope field is known and the depth of the film is standardized. The film is usually observed with the high power (x 40) dry objective of the microscope and the area of the high power field (HPF) so observed may be calculated from its diameter as measured by the use of a slide micrometer. Thus if the field diameter is 0.44 mm, the area of the HPF is 0.15 mm^2.

The depth of the wet film depends primarily on the volume of the drop of urine placed on the microscope slide and the area of the coverslip applied to it. Mix the urine sample carefully and then transfer 0.05 ml on to the middle of a microscope slide. At once apply a coverslip 22 x 22 mm in dimensions, avoiding trapped bubbles. The film should show a small excess of fluid along the edges of the coverstip and then be about 0.1 mm in depth. If the area of the HPF is 0.15 mm^2, the volume of urine observed in an HPF will be about 0.015 mm^3. Under these conditions the finding of 1 leucocyte per 7 high power fields corresponds with 10^4 leucocytes per ml and the finding of clearly larger numbers than this indicates significant pyuria.

Two sources of error in leucocyte counts have to be avoided. In cases of pyelonephritis or other kidney disease, care must be taken to avoid confusing tubular epithelial cells with leucocytes. In women, contamination from the vagina may introduce large numbers of leucocytes into a sample of voided urine; the presence of squamous epithelial cells along with pus cells in the sample is evidence that such contamination has taken place and the leucocyte count is then invalidated.

There is little value in microscopical examination of the wet film for bacteria. It may be difficult to recognize bacteria amid amorphous urate particles and it is scarcely possible to distinguish whether bacteria present in significant numbers consist of a single infecting species or a mixture of faecal or vaginal contaminants. Contamination from the vagina may lead to the presence of large numbers of lactobacilli which will not grow on the media used routinely for culture. If in cases where pyuria has been recognized an indication of the type of infecting bacterium is urgently required, a Gram film of centrifuged deposit may be examined, but the findings must be interpreted with caution.

Apart from the presence of leucocytes and bacteria in the wet film, note should be taken of the presence of squamous or tubular epithelial cells, tubular casts, red blood cells and crystals which may have diagnostic significance in non-infective conditions.

Semi-quantitative culture

Filter paper method

This method of semi-quantitative culture is rapid and very economical in the use of culture medium but growths are often confluent and if mixed, require to be plated out to obtain pure subcultures for identifying and sensitivity tests. A standard 6 mm wide strip of absorbent fluff less blotting or filter paper is bent into an L shape with a 12 mm long foot (area 12 x 6 mm) and sterilized at 160°C for 1 h. Dip the whole of the angulated end and foot into the mixed, uncentrifuged sample of urine, withdraw it and wait a few seconds to allow all the excess fluid to be absorbed into the paper. Then press the foot on to the surface of a marked section of a well-dried plate of agar culture medium. ensuring that the whole area of the foot makes contact with the medium. Remove the strip and discard it into disinfectant. Up to 8 or 10 samples can be tested in duplicate on different areas of a 9 cm plate. Incubate the plate and afterwards count the colonies growing on the impression area. Up to 50 colonies may be countable and heavier growths are noted as being semi-confluent (+) or confluent (++).

Estimate the number of viable bacteria per ml of urine from the count of colonies on the impression area or the pattern of semi-confluent or confluent growth. Very approximately, the value of 10^5 bacteria/ml corresponds to a count of 25 colonies of bacilli or *30* colonies of cocci. As, however, the porosity and adsorptive power of the papers obtained from different manufacturers often vary, calibration curves should be prepared from impression counts made in preliminary tests on diluted broth cultures for which viable counts are made in parallel.

Dip-slide method

The method of semi-quantitative culture on dip-slides or dip-spoons is the least laborious for the laboratory and as the medium is seeded with urine immediately it has been passed, obviates the difficulty of having to prevent bacterial multiplication during transport to the laboratory. It is especially convenient for the routine screening of large numbers of patients, e.g. in ante-natal or geriatric assessments, and for use in clinics and practices remote from the laboratory. Its disadvantages are that it does not provide material for microscopical examination for the cellular content of the urine and that when the bacterial count is high and the growth on the dip-slide confluent, it is difficult to judge whether the growth is pure or mixed and to obtain an unmixed inoculum's for identifying and sensitivity tests.

The dip-slide is a small plastic tray carrying a layer of an appropriate agar culture medium. Opposite sides of the tray may carry different media. e.g. CLED agar medium on one side and MacConkey, brain heart infusion or pseudomonas selective agar on the other. The slide is supplied in a Universal-type container, being held on a stalk fastened rigidly to the inside of the screw-cap of the container. Such outfits are available commercially (e.g. from Difco, Gibco, Oxoid and Roche).

A mid-stream specimen of urine is collected in a clean container. The cap of the dip-slide container is unscrewed and held while the dip-slide is withdrawn from the container and briefly immersed in the urine. On its removal, the excess urine is drained off by contact of the bottom of the slide with the wall of the urine vessel and the device is then returned into its container and the cap screwed on tightly. Alternatively an intelligent patient may be instructed to grasp the dip-slide by the cap and hold it in such a way as to immerse the agar tray in the stream of urine while the mid portion is being passed; then to shake off the excess urine and replace the device in its container.

The charged dip-slide in its sealed container is sent to the laboratory, the duration and temperature of transport not being critical. It is then incubated at 37°C overnight and examined for a growth of colonies. (Prolonged culture at 15-18°C often gives comparable results to those obtained by incubation overnight at 37°C.)

Where urine samples are collected in a clinic with the facility for incubation at 37°C, the dip-slide can be incubated there and examined next day by a doctor or nurse who has been familiarized with the appearances of growths. The clinician then gets an early indication of the results and as only the minority of dip-slides that show significant growth need be sent to the laboratory for identification and sensitivity tests, the laboratory is spared from undertaking the large amount of clerical work required for the reception and reporting of the negative specimens.

The Count of viable bacteria in the Urine is estimated approximately from the number of colonies or the pattern of semi-confluent or confluent growth seen on the medium on slide. Commercial suppliers of dip-slides provide charts showing representing numbers and patterns by comparison with which the viable count can be read. Otherwise, charts may be prepared in the laboratory from the findings in trials of the dip-slides in diluted broth cultures on which viable counts are obtained.

Quantitative Method

Standard loop method

An inoculating loop of standard dimensions is used to take up a small, approximately fixed and known volume of mixed uncentrifuged urine and spread it over a plate of agar culture medium.

The plate is incubated, the number of colonies counted or estimated, and this number used to calculate the number of viable bacteria per ml of urine. Thus if a 0.004 ml loopful of urine yields 400 colonies, the count per ml will be 10^5, just indicative of significant bacteriuria.

It is. however, much simpler to purchase standard. ready made sterile disposable (single-Use) plastic loops that will hold a fixed volume in the range 0.001-0.01 ml. When loops holding 0.004 ml or more are used and the loopful is spread uniformly over the whole of a plates colonies may be so numerous as to be confluent and there may be no single, separate colonies available for picking and the preparation of pure subcultures. Accordingly, it is usual to spread these larger loopfulls over only a sector of the plate and streak out from that sector over other sectors to ensure the production of separate colonies. The approximate number of viable bacteria may then be estimated from the number of colonies on the plate or the weight of confluent or semiconfluent growth on the different sectors according to a prearranged scheme.

Identification and sensitivity tests

If similar colonies are found in numbers suggesting significant bacteriuria, a separate colony or a portion of apparently pure growth should be subcultured for identification and testing of its sensitivity to antibiotics.

The appearance of the primary growth on CLED or MacConkey medium will suggest the kind of organism that is present. How much more precisely it should be identified is a matter for consideration. Probably coliform bacilli should be differentiated into *E. coli, Klebsiella, Proteus. Pseudomonas* and other 'coliforms'; *S. saprophyticus* and *S. aureus* should be distinguished from other *Staphylococci*, and *Enterococci* should be distinguished from other *Streptococci*.

Detailed characterization and typing of isolates may be done in epidemiological studies of cross-infection and in cases where it is important to distinguish between reinfection of a patient with a new strain and relapse of infection with a strain that was formerly present.

Antibiotic sensitivity tests arc best done with an appropriately diluted inoculum of a pure subculture, but if prior microscopy has indicated that infection may be present, primary sensitivity tests may be set up at the same time as the initial culture by flood-

inoculating the urine on to a suitable medium drying the surface and applying sensitivity test disks.

As antibiotics are concentrated in urine to higher levels than are found in the tissues, high-content test disks should be used.

If the patient is attending a general practice or outpatient clinic, drugs suitable for oral administration should be tested. e.g. amoxycillin or ampicillin (25 µg disk), cephalexin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), nitrofurantoin (50 µg), and trimethoprim (2.5 µg). Tests with disks containing amoxycillin (20 µg) with clavulanate (10 µg) marketed as Augmentin (30 µg) may be done as a routine on a separate plate with a β-lactamase producing strain of E. coil as control organism. but results should not be issued unless resistance to ampicillin/ainoxycillin alone is encountered. Tests with disks containing trimethoprim and sulphamethoxazole in combination are not recommended; if an organism is resistant to trimerhoprim alone, the use of cotrimoxazole would be inappropriate.

For patients in hospital. For whom parenteral therapy might be appropriate, sensitivities to cefuroxime (30 µg) and gentamicin (10 µg) should also be tested, and when resistant pseudomonas is present, sensitivity to amikacin, netilmicin, tobramycin, azlocillin, ceftazidime, and ticarcillin may be tested.

It is generally of little value to identify the species in the mixed cultures of stool -type bacteria that are commonly obtained from patients with indwelling catheters. Antibiotics are of little use in the treatment of such infections. The findings may be reported as ‘many mixed bacteria’ and the patient’s bladder may be treated by irrigation with a mild disinfectant such as Hibitane or Noxythiolin (without specific sensitivity testing).

9. PYREXIA OF UNKNOWN ORIGIN (PUO)

The term PUO is generally applied to any febrile illness lasting more thanone week, the cause of which is obscure due to the absence of obvious specific or localizing signs and symptoms in these conditions there is thus a special need for a laboratory diagnosis to guide the choice of appropriate therapy.

Etiology:

About two-thirds of the more acute cases and one-third of the chronic cases are due to infections of one kind or another and the remainder of cases is due to non-infective causes such as leukaemia, early carcinoma, collagen diseases, sarcoidosis, Crohn's disease and drug reactions.

Many kinds of infections may present as PUO including many common infections that in some patients fail to show their usual diagnostic features.

They include: (1) urinary tract infections; (2) lung, subdiaphragmatic, appendicular and other deep abscesses; (3) septicaemia as, for example, that associated with cryptic abscesses, pneumonia, pyelonephntis, biliary tract infection, infective endocarditis and immunodeticiencies; (4) enteric fever, tuberculosis, brucellosis, syphilis, non-meningitic meningococcal infection, rheumatic fever, leptospirosis without jaundice or meningitis, Q fever and toxoplasmosis; (5) many viral infections, e.g. infectious mononucleosis, rubella and other infectious fevers without their typical rashes; (6) malaria and, less frequently, leishmaniasis, trypanosomiasis and other tropical infections in travellers returned from abroad, and (7) helminthic infestations.

Very careful history taking is an essential preliminary to diagnosis. Enquiry must be made about foreign travel, occupation, contact with cases of infectious diseases and contact with animals. The physician must inform the microbiologist of any such relevant facts.

Laboratory diagnosis of PUO infections

The following procedures should be considered. Tests should first be done for the more likely infections and then, if these are negative, tests for the less likely should be done. For fuller details, refer to the chapters dealing with the specific causal organisms.

1. Blood culture should always be attempted. A first specimen should be collected before antibiotics are given, and several specimens collected on separate occasions should be examined before a negative result is accepted.
2. Specimens of urine, throat secretion, sputum (if present) and stool should he examined for the common pathogenic bacteria, and faeces should be examined for protozoa, cysts and helminthes ova.
3. Paired sera should be collected for serological tests for antibody responses to a range of possible pathogens. e.g. cytomegalovirus, hepatitis B virus, influenza virus, infectious mononucleosis virus, Chlamydia, coxiella, Ricketsia, Mycoplasma, Brucella, Legionella, Leptospira Syphilis spirochaete, toxoplasma, aspergillus and other fungi, and entamoeba, Salmonella typhi (Widal test with diagnostic titre I/80 0r 4 folds increase within one week). The antistreptolysin-O (ASO) test should be done for cryptic S. *pyogenes* infection. The first specimen should be taken as early in the illness as possible and the second 2-4 weeks later. IgM for single sample in infection like

Toxoplasma or hepatitis A virus are enough, while for other infections rising titer are required.

4. Hematological investigations should be done to detect leucocytosis, suggestive of a cryptic abscess; eosinophilia, suggestive of helminthiasis; and atypical 1ymphocytes , suggestive of infectious mononucleosis.

5. A tuberculin test and a chest X-ray shoul be done to detect tuberculosis.

6.Thick and thin blood fillms Should be examined for malaria, leishmaniasis, trypanosomiasis and filariasis in travellers returned from countries in which these infections are present.

Children Specific Infections

Infants and children can be affected by any of previous infections. In addition, there are usually specific infections affecting mainly children.

-Croups.

-Exanthematous. They are infections with prodromal stage of fever followed by specific rash. The common types are due to measeles, German measeles, Chicken pox and some add scarlet fever.

- Whooping cough caused by Gram-negative bacilli Bordetella pertusis cultured on specific media Border-Gengou or Lacey s mediawith antibiotic like penicillin, rising complement fixation test is also diagnostic with absolute lympocytosis in blood picture.

-Diphteria.

Clinical Groupings for Fungal Infections

The following clinical groupings may be recognized:

Skin Mycology

The Superficial Mycoses

These are superficial cosmetic fungal infections of the skin or hair shaft. No living tissue is invaded and there is no cellular response from the host. Essentially no pathological changes are elicited. These infections are often so innocuous that patients are often unaware of their condition.

Disease	Causative organisms	Incidence
Pityriasis versicolor Seborrhoeic dermatitis including Dandruff and Follicular pityriasis	*Malassezia furfur* (a lipophilic yeast)	Common
Tinea nigra	*Exophiala werneckii*	Rare
White piedra	*Trichosporon beigelii*	Common
Black piedra	*Piedraia hortae*	Rare

The Cutaneous Mycoses

These are superficial fungal infections of the skin, hair or nails. No living tissue is invaded, however a variety of pathological changes occur in the host because of the presence of the infectious agent and its metabolic products.

Disease	Causative organisms	Incidence
Dermatophytosis Ringworm of the scalp, glabrous skin and nails.	Dermatophytes (*Microsporum, Trichophyton, Epidermophyton)*	Common
Candidiasis of skin, mucous membranes and nails.	*Candida albicans* and related species.	Common
Dermatomycosis	Non-dermatophyte moulds *Hendersonula toruloidea* *Scytalidium hyalium* *Scopulariopsis brevicaulis*	Rare

The Subcutaneous Mycoses

These are chronic, localized infections of the skin and subcutaneous tissue following the traumatic implantation of the aetiologic agent. The causative fungi are all soil saprophytes of regional epidemiology whose ability to adapt to the tissue environment and elicit disease is extremely variable.

Disease	Causative organisms	Incidence
Sporotrichosis	*Sporothrix schenckii*	Rare
Chromoblastomycosis	*Fonsecaea, Phialophora, Cladosporium* etc.	Rare
Phaeohyphomycosis	*Cladosporium, Exophiala, Wangiella, Bipolaris, Exserohilum, Curvularia*	Rare
Mycotic mycetoma	*Pseudallescheria, Madurella, Acremonium, Exophiala* etc.	Rare
Subcutaneous zygomycosis (Entomophthoromycosis)	*Basidiobolus ranarum Conidiobolus coronatus*	Rare
Subcutaneous zygomycosis (Mucormycosis)	*Rhizopus, Mucor, Rhizomucor, Absidia, Saksenaea* etc.	Rare
Rhinosporidiosis	*Rhinosporidium seeberi*	Rare
Lobomycosis	*Loboa loboi*	Rare

Dimorphic Systemic Mycoses

These are fungal infections of the body caused by dimorphic fungal pathogens which can overcome the physiological and cellular defenses of the normal human host by changing their morphological form. They are geographically restricted and the primary site of infection is usually pulmonary, following the inhalation of conidia.

Disease	Causative organisms	Incidence
Histoplasmosis	*Histoplasma capsulatum*	Rare*
Coccidioidomycosis	*Coccidioides immitis*	Rare*
Blastomycosis	*Blastomyces dermatitidis*	Rare*
Paracoccidioidomycosis	*Paracoccidioides brasiliensis*	Rare*

*more common in endemic areas.

Opportunistic Systemic Mycoses

These are fungal infections of the body which occur almost exclusively in debilitated patients whose normal defense mechanisms are impaired. The organisms involved are cosmopolitan fungi which have a very low inherent virulence. The increased incidence of these infections and the diversity of fungi causing them, has parallelled the

emergence of AIDS, more aggressive cancer and post-transplantation chemotherapy and the use of antibiotics, cytotoxins, immunosuppressive, corticosteroids and other macro disruptive procedures that result in lowered resistance of the host.

Disease	**Causative organisms**	**Incidence**
Candidiasis	*Candida albicans* and related species.	Common
Cryptococcosis	*Cryptococcus neoformans*	Rare/Common
Aspergillosis	*Aspergillus fumigatus* etc.	Rare
Pseudallescheriasis	*Pseudallescheria boydii*	Rare
Zygomycosis (Mucormycosis)	*Rhizopus, Mucor, Rhizomucor, Absidia* etc.	Rare
Hyalohyphomycosis	*Penicillium, Paecilomyces, Beauveria, Fusarium, Scopulariopsis* etc.	Rare
Phaeohyphomycosis	*Cladosporium, Exophiala, Wangiella, Bipolaris, Exserohilum, Curvularia.*	Rare

Obstetric, Perinatal and neonatal infections

INFECTIONS IN PREGNANCY

Infections in pregnancy may cause spontaneous abortion. premature labour, still birth, perinatal or congenital infection. The risks to the fetus or *newborn* infant from infection are far greater than the risks to the mother since the incidence of serious maternal infections has greatly declined during the last 40 years. Much larger numbers of low birth weight infants are born each year compared with even 20 years ago. These now survive with the help of special care baby units. However, they are highly susceptible to serious infection because of their immature phagocvtic, humoral and cellular immune defence mechanisms. The 1gM immunoglobulin starts to be produced from about 20 weeks of fetal life, but IgG immunoglobulin is entirely derived from the mother by the transplacencal route.

infections in early pregnancy

Spontaneous abortion can result following maternal infection due to many viruses including rubella, influenza, mumps, measles and Coxsackie A16 viruses. Bacterial and protozoal infections, such as toxoplasmosis, only rarely cause abortion in Britain.

Asymptomatic bacteriuria of pregnancy occurs in about 5% of pregnant wome2attending the antenatal clinic where the urine should he routinely screened in early pregnancy. These infections can develop into severe pyelitis of pregnancy in up to about one-third of the cases unless promptly treated

Intra-uterine infections due to the -TORCH' group of organisms *(Toxoplasma gondii,* rubella virus. cytomegalovirus and herpes simplex virus) and also varicella-zoster virus can result in congenital malformations especially when infections occur in early preg-nancy. Organogenesis is completed mainly during the first trimester and *maximum* embryological development occurs in the first 6 weeks of pregnancy. The earlier that infection occurs, the more likely it is that multiple serious congenital malformations will result. Rubella virus can also cause selective congenital defects in later pregnancy, between the fourth and sixth months, such as eighth cranial nerve damage leading to deafness. These intra-uterine infections, and also congenital syphilis, are described in a section on congenital infections below.

Intra-uterine infection with HIV can occur either in early or late pregnancy when the mother has HIV infection — resulting in AIDS in some children *(see below).*

Table 8.1. Perinatal and neonatal bacterial infections

Main organisms	Neonatal infection			Routes of infection include			
				Pregnancy		Labour and delivery : From genital tract/perincum	Postnatal From other neonates, hospital staff and environment
	Early onset	*Late onset*	*Main sites infected include*	*Transplacental*	*Asccnding*		
E. coli, Klebsiella and other Gram- negative bacilli	+		Blood, lungs, umbilical stump		+	+	
		+	Blood, menings, umbilical stump, urinary tract			+	+
Enteropathogenic strains of E. coli: Salmonella, Campylobacter, Shigella		+	Gastrointestinal tract and blood			+	+
Lancefield group B streptococci	+		Blood, lungs, umbilical stump		+	+	
		+	Meninges, blood			+	+
Staph. aureus		+	Skin, umbilical cord, eye, blood, bone and joints, lungs				+
Neisseria gonorrhoeae	+		Eye			+	
Treponema Pallidum	+	+	Multi–system (see Congenital)	+			
Listeria monocytogenes	+		Blood, lungs, meninges		+	+	

Early onset during first 4 or 5 days of life.
Late onset after 4 or 5 days .

Infections in late pregnancy

The fetus or neonate is at risk when intra-uterine infections are acquired in late pregnancy either by the transplacental route or b ascending infection from the maternal genital tract. Intra-uterine infections that max' be acquired by the transpiacental route include varicella-zoster, hepatitis B, Coxsackie B *and* parvovirus B19 infections. Parvovirus acquired in the second or third trimester can cause hydrops foetalis with consequent fetal loss.

Intra-uterine infections acquired by the ascending route include those due to bacteria colonizing the vagina or perineum, such as Lancefield group B haemolytic streptococci, E. coil, *Kiebsidlla,* Proteus, *Bacterozdcs* and staphylococci. Other infections acquired by this route include those due to *Listeria monocytogenes,* herpes simplex virus and *Mycoplasma hominis.* Chorioamnionitis due to these organisms may cause a maternal pyrexial illness possibly associated with positive blood or high vaginal cultures, and the pregnancy then often ends prematurely with the delivery of either a stillbirth or a sick neonate who may have acute respiratory distress. The risks of chorioamnionitis developing are greatly increased when the membranes have been ruptured for more than 24 – 48 h. The range of organisms that may cause infections in association with prolonged ruptured membranes is so vast that rational guidance for effective antibiotic prophvlaxis in this situation is difficult.

PERINATAL INFECTIONS

The effects on the fetus of perinatal infection are likely to he greatest when the pregnancy has not reached full term, especially between weeks 26 and 36. Perinatal infection can result from an infection acquired in late pregnancy or during labour and delivery. The consequences of perinatal infection for the infant are likely to be more serious when the birth weight of the infant is less than l000 —1500 g. The organisms that can infect the infant during birth from the genital tract or perineum are similar to those already mentioned for later intra-uterine infection via the ascending route.

In addition, there *are* some sexually transmissible organisms that may cause infection including *Neisseria gonorrhoeae, Chlamydia trachomatis* (TR1C) agent and herpes simplex type II

The Lancefield group B haemolytic streptococcus (also known as Strep. *agalactiae)* has increasingly been found to cause perinatal infections during the last 20 years when larger numbers of low birth weight infants have been delivered. Lancefield group B streptococci and *E. coli* together cause more sepsis and deaths in infants during the first week of life than any other organisms. The highest perinatal mortality rate, about 43%, is associated with serious sepsis due to Lancetield group B streptococci. Early onset infections due to Lancefield group B

streptococci and Gram-negative bacilli arc characterized by septicaemia and typically the infective process involves many parts of the body including the lungs and umbilical cord stump. The neonate shows signs of being unwell, even during the first 24 h of life. Early onset Lancefield group B streptococcal infection may sometimes present as acute respiratory distress syndrome.

NEONATAL AND CONGENITAL INFECTIONS

Neonatal infections due to Lancefield **group** *B haemolytic streptococci and Gram-negative bacilli*

Early onset infections due to Lancefield group B streptococci and Gram-negative bacilli have been described above in perinatal infections. After 4 – 7 days of life, 'late onset' neonatal infections due to these organisms may appear. The source of the organisms in late onset infections need not necessarily be the maternal perineal or genital tract flora. The source may he the already infected neonates in a baby unit or the hands of hospital staff which transmit these infections from one neonate to the next. Occasionally moist contaminated equipment, such as baby incubators which are humidified or baby resuscitation equipment, is the source of infection in a common source outbreak due to *Pseudomonas aeruginosa* or other Gram-negative bacilli. Late onset serious Lancefield group B streptococcal or Gram-negative infection is often characterized by the development of meningitis.

Neonatal staphylococcal infections

Staphylococcus aureus is a frequent cause of minor neonatal sepsis and occasionally is associated with more serious infections. During the 1950s and early 1960s, this organism was much more often the cause of moderately severe sepsis and occasionally caused fatal staphylococcal pneumonia or septicaemia. Outbreaks of neonatal sepsis were common in nurseries due to phage group I *Staph. aureus* strains, such as the '80/81' phage type strain, which were characteristically penicillin resistant. These outbreaks have become much less evident during the last 20 years. When they occur phage typing of the epidemic strains, which are usually resistant to penicillin only, shows phage types other than the 80/81 type. It seems probable that there has been a decline in the virulence of *Staph. aureus* strains, but other factors to control and treat staphylococcal sepsis in nurseries have also contributed to the reduced incidence of staphylococcal infections. More than 30% of neonates become colonized normally by *Staph. aureus* during the first week of life. The first sites to become colonized include the umbilicus, groin, nose, axillae and wrists.

One advantage of encouraging close contact between the mother and infant during the first 24-48 h after birth is that the infant may become colonized with a staphylococcus that the mother might be carrying rather than a hospital nursery staphylococcus. Handling of the neonate by the hospital staff should be reduced to a minimum.

Clinical features of neonatal *Staph. aurcus* infections include multiple skin pustules appearing after the third day of life, 'sticky' eye, and less often an infected umbilical stump or breast abscess. More serious staphylococcal infections are uncommon and include pneumonia, osteomyelitis, septic arthritis, and septicaemia. Other serious

staphylococcal neonatal problems include pemphigus neonatorum and the scalded skin syndrome, but these are fortunately rare. The latter syndrome, also known as Ritter's disease, is produced by a toxin that is secreted by certain phage group U strains of *Staph. aureus,* such as some type 71 strains, and the toxin splits the epidermis causing 'toxjc epidermal necrolysis'. Outbreaks of Ritter's disease in nurseries can be associated with a high neonatal mortality rate. A frequent maternal complication of neonatal colonization of the nose by a hospital nursery strain of *Staph.. aureus* is breast abscess. The organism may enter a traumatized nipple during breast feeding and the mother may not present with the breast abscess until many days or weeks after the birth of the infant.

Treatment of minor pustules with systemic antibiotics is not indicated but topical application of anti-staphylococcal substances such as 'triple dye' or hexachorophane may be useful. When more marked sepsis occurs, such as sticky eye or umbilical sepsis, swabs should be collected. For more serious illness, blood cultures are necessary before the start of prompt 'blind' treatment with systemic cloxacillin or flucloxacillin. Virtually all the staphylococcal strains are resistant to penicillin but sensitive to cloxacillin. If an outbreak is suspected the strains of *Sraph. aureus* isolated may be sent to a reference laboratory by the microbiologist for phage typing. Isolation of infected infants is important and in the event of an outbreak the unit may have to be temporarily closed.

Prevention of neonatal staphylococcal infections is more likely to be achieved in nurseries that are not overcrowded and where the hospital staff use good hand-washing and aseptic techniques. Further useful protection for normal birth weight infants is possible by applying hexachiorophane powder routinely to the umbilicus, groins and axillae daily from birth.

Other neonatal infections

These include urinary tract infections, gastroenteritis and meningitis. *E. coli* is the most common causative organism.

Neonatal gastroenteritis

Gastroenteritis is an important infection in both the neonatal period and throughout infancy since serious water and electrolyte depletion may result with possibly fatal consequences; the problem is particularly great in the less developed world today. The causative organisms in neonates include enteropathogenic strains of *E. coil* or toxigenic strains of *E. coli, Salmonella* and *Gampvlobacter.* In contrast, rotaviruses do not usually appear to cause clinically significant gastroenteriris episodes in neonates, although they are important in later infancy. Dangerous outbreaks of gastroenteritis may occasionally occur in maternity units and nurseries

due to epidemic strains of the above bacteria. Salmonella outbreaks have sometimes started after a mother has been admitted with asymptomatic convalescent carriage of a food-poisoning strain of *Salmonella,* such as *Salmonella enteruidis.* Her infant ma~ develop salmonella gastroenteritis soon after birth and the infection may he transmitted by the hands of staff to other neonates in the unit. It is not practical to screen all pregnant mothers for salmonella carriage when they are admitted to a maternity unit.

Breast feeding

As with other infections the effects of gastroenteritis are particularly serious in low birth weight neonates and some protection against infection should be given through the administration of fresh human breast milk. Breast feeding rather than the feeding of artificial milk in a bottle should be routinely encouraged to give the neonate some protection against infections, particularly gastroenteritis. Human breast milk contains immunoglobutins, especially IgA, which in conjunction with the other constituents of complement, transferrin, and lysozvme, are active against Gram-negative bacilli. The high lactose content of human milk also encourages the growth of lactobacilli which results in acid faeces and an intestinal environment which discourages the multiplication of any strains of pathogenic *E.* ***coli which*** may be introduced. In some centres, pooled expressed breast milk is administered to low birth weight infants only after it has been gently pasteurized to remove any contaminating potential pathogens, such as *Klebsiella,* without destroying the antibacterial constituents; in recent years there has been an additional reason for pasteurizing milk to inactivate any contaminating HIV present to reduce the small risk of transmission of AIDS virus *via* pooled expressed milk

Microbiological investigations

Many infected neonates develop only non-specific clinical features possibly suggestive of infection, such as lethargy, excessive weight loss or failure to regain birth weight, poor feeding, irritability, vomiting, jaundice, cyanotic spells or bloody diarrhoea. In infants with these features, prompt bacteriological investigations before the start of an immediate antibiotic treatment are essential.

Investigations should include the taking of blood cultures, swabs of umbilicus, skin, eye or any septic site, faeces for culture, and the microscopy and culture of urine and cerebrospinal fluid. When pus is present, a Gram—stain of this can frequently give a rapid indication of the likely causative group of organisms; predominant numerous Gram-positive cocci could suggest the possibility of a Lancefield group B haemolvtic streptococcus or *Staphylococcus aureus* infection. However, the results of microscopy and culture of skin sites are often difficult to

interpret in practice since colonization of the neonatal skin by streptococci, staphylococci and Gram-negative bacilli is also common and difficult to distinguish in the laboratory from infection due to these same organisms.

Antibiotic treatment of serious neonatal infections

As soon as the cultures have been collected a combination of benzylpenicillin plus gcntamicin (or netilmicin) should be urgently started as empirical treatment for neonates with suspected serious 'early onset' infections with Lancetield group B streptococci or Gram-negative bacilli.

Special baby care units which accommodate low birth weight infants often have cross-infection problems causing 'late onset' infections with multiple antibiotic—resistant strains of *E. coli, Kiebsiella aerogenes, Pseudomonas* or other Gram-negative bacilli. However, gentamicin would usually also be suitable for treating these infections although penicillin should be added in case of late onset Lancefield group B streptococcal *infections.* The antibiotic sensitivitv patterns of bacterial strains encountered in each hospital unit must be taken into account when *determining* a blind antibiotic treatment policy. Gentamicin-resistant or netilmicin-resistant strains of Gram-negative bacilli are now becoming more common in a few hospitals and, in this situation, the use of one of the cephalosporins such as ceftazidime may he indicated. Ceftazidime has the advantage of effective activity against gentamicinresistant *Pseudoinonas oernginosa* but there is some uncertainty that it provides a good clinical effect against grour B haemolytic streptococcal infection and penicillin should be added if mixed streptococcal and Gram-negative infection is suspected. *Pseudounmas aerug— mosa* infections are infrequent in most hospital neonatal units, but, when they occur, they are often associated with a high mortality rate unless effectively treated in the early stages. Cloxacillin is only necessary for blind therapy if *Staph. aureus* infection is suspected on clinical or epidemiological grounds.

If meningitis is suspected from abnormal CSF microscopic findings in a sick neonate, 'blind' treatment with chloramphenicol may he considered, although high dosage benzylpenicillin should also be started if Gram-positive cocci (possibly Lancefield group B streptococci) were seen in the Gram-stained CSF deposit. The dosage of chioramphenicol can be adjusted so that the grey baby syndrome does not occur while giving effective levels of drug against the infecting organism. Assays of chloramphenicol in CSF and serum are helpful. Most authorities have pointed out the problems associated with chioramphenicol of drug toxicity, unpredictable drug concentrations and a significant minority of Gram-negative bacilli resistant to chioraniphenicol. Instead these authorities prefer to recommend a later generation cephalosporin such as

cefotaxime, ceftriaxone or ceftazidime. However, some Gram-negative bacilli are resistant even to these cephalosporins and initial 'blind' antibiotic therapy of meningitis with a combination of cephalosporin plus an aminoglycoside such as gentamicin should be considered. \When cultures show that the causative organism of meningitis is fully sensitive to the cephalosporin alone the aminoglycoside may be discontinued.

Rational specific antibiotic treatment of serious neonatal infections only becomes possible once a microbiological diagnosis has been made and the antibiotic sensitivity test results on the infecting pathogen(s) are known. When gentamicin or other aminoglycoside drugs are used, serum assays of the antibiotic are desirable to check that effective levels are achieved and to adjust the dosage according to the results of assay.

Necrotizing enterocolitis

This condition is rare and occurs mainly in low birth weight neonates following intensive care management in special care baby units. The infant with necrotizing enterocolitis (typically a pre-term baby in its second or third week of life) presents with vomiting, abdominal distension and blood in the faeces. A straight X-ray of the abdomen shows initially a thickened bowel wall and then signs of gas in the wall of the bowel (pneumatosis intestinalis) or within the portal venous system. In severe cases, the child is shocked with septicaemia and peritonitis. Findings at operation or post mortem are ischaemic necrosis and ulceration of the bowel wall particularly in the ileocaecal region.

The cause is not known, although it seems likely that a microbial agent is one of the aetiological factors. Various anaerobes ma play a role, particularly *Glostridium* species, but this is uncertain. Epidemics have sometimes occurred suggestive of a microbial cause.

Treatment is empirical and includes intravenous fluids, gastric suction and a combination of penicillin, metronidazole and genramicin. Surgery on the bowel is also sometimes necessary. The condition is frequently fatal.

Prevention of neonatal infections

General measures to reduce cross-infection in neonatal units have already been mentioned briefly, the main measure being frequent hand washing by staff and the use of good aseptic techniques. For high risk areas, such as special care baby units, an antiseptic preparation for the hands of staff, such as an alcoholic chlorhexidine solution or chiorhexidine detergent, is recommended. The usefulness of human breast milk has been discussed above.

The use of antibiotic prophvlaxis in pregnancy to prevent group B haemolyric streprococcal meningitis and septicaemia in neonates

Specific preventative measures for *infections* that have not been so far discussed include precautions against hepatitis B, gonococcal ophthalmia neonatorum, herpes simplex infection and varicella-zoster.

Anti-hepatitis B immunoglobulin may be necessary for neonates born to mothers who have had hepatitis B around the time of delivery and some mothers who are HBsAg positive. This immunoglobulin needs to be given to the infant during the 4S h after delivery to he effective in the prevention of liver disease (see Chapter 16). Active immunization with genetically engineered hepatitis B vaccine is also indicated in infants at risk.

Gonococcal ophthalmia was prevented by the instillation of silver nitrate eye drops soon after birth but this measure is not worth while today as the condition is relatively rare. However, when a mother is known to have had recent gonorrhoea, this measure could be considered in addition to antibiotic treatment of the maternal infection.

Herpes simplex type II is a common cause of infection and ulceration of the female genital tract but is only rarely associated with serious disseminated disease, encephalitis or hepatitis in infants in Britain. Serious neonatal herpetic disease has been mainly reported from the USA. However, when genital herpes simplex infection is apparent, especially primary infection, near the time of delivery the protection of the neonate against herpetic infection should be considered by performing a caesarean section. This method of delivery of the infant reduces the chance of infection but does not eliminate this possibility altogether Most neonatal units treat the infant prophylactically with acyclovir.

Varicella-zoster infection in pregnancy may cause severe VZ pneumonitis in the mother and disastrous neonatal infection. If a seronegative mother has contact with a case of chickenpox or shingles she should be given hyperimmune anti-varicella-zoster immunoglobulin (ZIG) as soon as possible after the contact. The neonate should also receive ZIG if maternal varicella has an onset during the 7 days before or after delivery as there will be no transfer of maternal antibodies when infection occurs then. When varicella infection occurs between 8 and 20 weeks of pregnancy, there is a 2% risk of fetal embrvopathy with possible consequences of microcephaly, mental retardation, eve and other lesions. It is not known if *ZIG* or prophylaxis with aciclovir administered to the mother in early pregnancy has a significant protective effect for the fetus. Aciclovir is not licensed for use in pregnancy. However, its use should especially be urgently considered if clinical features of varicella appear in either the pregnant mother, post term mother or neonate to reduce the risk of possibly fatal complications.

Congenital infections

Fetal infection due to *Treponema pallidum,* rubella virus. cytomegalovirus. HIV, *toxoplasma gondii* and varicella-zoster virus may follow intra-uterine infection. Transplacental spread of the organisms to affect the fetus may occur when there has been maternal blood stream infection (as described in Infections in pregnancy *above).* Once fetal infection occurs due to these organisms, there are possible consequences of an abortion, stillbirth or congenital infection. These and other congenital infections are included in *Table 8.2.* Variceliazoster infection is discussed above. HIV infections are preventable in the infant

Congenital syphilis.

Treponema pallidum can affect many different sites of the body. The clinical features may appear early in neonatal life or later, often between the ages of 5 and 15 years. With the advent of routine antenatal serological screening for syphilis and treatment with penicillin this condition has become very rare in Western populations today.

Clinical features in the neonate may include skin lesions such as a generalized maculopapular rash, raised moist mucocutaneous lesions, 'snuffles', hepatosplenomegaly, lymphadenopathy, periostitis, osteochondritis and failure to thrive.

Later presentations of congenital syphilis may include interstitial keratitis. scquelae of central nervous system infection, such as mental deficiency and eighth nerve deafness, anterior ribial periostitis, gummata in skin and mucous membranes, and Hutchinson's teeth (peg-shaped upper permanent incisors), bone and cartilage destruction, leading for example to a saddle-shaped nose.

Organisms Causing Congenital Infections

Main causative organisms include	***Associated infection***	***Routes of infection include***	
		Intra – uterine infection via transplacental route?	***Organisms from mother shortly before* or during delivery?***
Treponema pallidum	Congenital syphilis	Yes	
Rubella virus	Congenital rubella	Yes	
Cytomegalovirus	Congenital cytomegalovirus disease	Yes	
Toxoplasma gondii	Congenital toxoplasmosis	Yes	
Hepatitis B	Congenital hepatitis B infection	Yes (possible)	Yes (usually)
Herpes simplex (type II usually)	Neonatal herpes simplex infection		Yes
Varicella – zoster virus	Congenital varicella infection	Yes	Yes
Human immunodeficiency virus	Congenital AIDS	Yes	Yes
Listeria monocytogenes	Listeriosis		Yes
Neisseria gonorrhoeae	Ophthalmia neonatorum		Yes
Chlamydia trachomatis (TRIC) agant)	Ophthalmia neonatorum and neonatal pneumonia		Yes
Candida albicans	Congenital oral thrush		Yes

* Viruses, such varicella – zoster, may also infect fetus at this time via transplacental route.

Congenital rubella

Rubella occurring during the first month of pregnancy causes fetal damage in 60-80% of cases, while in the third month this risk falls to 10-20%, and in the fourth month to less than 10%. Multiple congenital defects usually result from rubella infection occurring during the first 10 weeks of pregnancy. An isolated congenital eighth cranial nerve lesion is a complication of rubella infection occurring at about the sixteenth week of pregnancy.

Characteristic clinical features of congenital rubella include cataracts and other ocular defects, eighth cranial nerve deafness, congenital heart lesions, such as patent ductus arteriosus and ventricular septal defect, low birth weight, failure to thrive, hepatosplenomegaly, thrombocytopenic purpura, microcephaly and mental retardation. Unfortunately, there is a continuing incidence of congenital rubella in Britain today; there are some susceptible women who should be offered rubella immunization.

Congenital cytomegalovirus disease

Cytomegalovirus is excreted in the urine by about 3% of apparently healthy pregnant women and by about 1% of newborn infants. Most babies with congenital infection show no harmful effects but serious disease may result in a minority of cases, especially of the central nervous system. Cytomegalovirus is a much more frequent cause of severe mental retardation than rubella or toxoplasmosis.

Clinical features of congenital cytomegalovirus infection may include jaundice, hepatosplenomegaly, thrombocytopenic Purpura, anaemia, spasticity, microcephaly, ophthalmic defects and mental retardation.

Congenital toxoplasmosis

Most infants with congenital toxoplasma infection have no clinical features, or only mild illness, shortly after birth, but many of these infants will develop choroidoretinitis later in life. Classical features of congenital toxoplasmosis characteristically include choroidoretinitis and encephalitis-causing neonatal convulsions, jaundice, hepatosplenomegaly and microphthalmia. In Britain there are about 12 severely affected children reported each year.

When classical features occur there is a 10% mortality rate approximately, but survivors often show serious sequelae which may include hydrocephalus, intracranial calcification, mental retardation and impaired vision.

Microbiological investigations

In practice, serological investigations are usually of greater value than attempts at isolation of the organism causing congenital infection, although both methods may be attempted in individual patients. Three general principles apply to serological diagnosis of congenital infections:

1. Maternal and infant serum samples should he tested simultaneously as soon as possible after birth and the antibody investigations should be repeated at appropriate intervals. When there is no active congenital infection, the infant's antibody titres progressively fall during the first few months of life, compared with the mother's antibody titres, due to the gradual disappearance of maternal antibody from the infant's serum. However, if there is active congenital infection the antibody titres of the infant may not fall during the first month of life and instead rising titres may be demonstrated.
2. *IgM* immunoglobulin determinations performed on the neonate's serum may give valuable evidence of congenital infection. The total 1gM fraction may be raised, but of greater diagnostic importance is the finding of raised specific 1gM antibodies against rubella, *Treponema pallidum,* cytomegalovirus or *Toxoplasma.* IgM tests are not helpful for HIV diagnosis, and HIV molecular biology tests may be indicated. In selected patients with evidence of primary toxoplasmosis in pregnancy a PCR test for *Toxoplasma* can be carried out on amniotic fluid.
3. Routine screening tests for the detection of the appropriate antibody should be performed in the first instance on the mother's and infant's sera. If these tests are negative there is little point in proceeding with further serological investigations against these organisms.

Treatment and prevention of congenital infections

Congenital syphilis can he treated effectively by a course of parenteral penicillin. Termination of the pregnancy must be considered when rubella is acquired during the first trimester.

Routine antenatal serological screening for syphilis, hepatitis B antigen carriage, immunity to rubella, and HIV (with consent) should be encouraged. When syphilis is discovered, penicillin is given to prevent congenital infection.

Rubella immunization is recommended for both infant boys and girls, to reduce the overall prevalence of rubella, and to prevent congenital rubella by ensuring that women are immune before pregnancy occurs. MMR vaccine is administered to infants with booster doses in later childhood. Rubella vaccine is also recommended for non-pregnant seronegative fertile females who must avoid pregnancy for at least 3 months following immunization.

Congenital toxoplasmosis cannot easily be prevented at present, although maternal toxoplasmosis should be treated where possible with spiramycin and occasionally termination of pregnancy is considered. In Britain the Department of Health does not currently recommend routine serological screening tests for toxoplasma during pregnancy, and most primary infections are asymptomatic, so it is difficult to detect primary infection. The risk of the latter to the fetus depends on the stage of

pregnancy, starting with 25% approximately in the first trimester and rising to 60% risk of fetal infection in the third. However, congenital malformations are most likely to follow infection in the first trimester, with a 75% chance of malformations. Pregnant mothers need to avoid handling cat litter and eating undercooked meat.

PUERPERAL SEPSIS AND **POST-PARTUM PYREXIA**

Puerperal sepsis, caused mainly by *Streptococcus pyogenes,* was a major cause of maternal deaths in the nineteenth and early twentieth centuries. About 100 years ago, Semmelweis showed how hospital attendants could spread the infection by their hands, and his observations helped to lay the foundations of good aseptic and antiseptic techniques in obstetrics.

The disease has fortunately become rare, although a few avoidable deaths in young mothers due to streptococcal puerperal sepsis have occurred in Britain during recent years. *Streptococcus pyogenes* (Lancefield group A haemolytic streptococcus) has the ability to spread rapidly in the soft tissues of the genital tract and can cause a fatal septicaemia within 24-48 h of the start of the illness. The source of the *Strep. pyogenes* infection could be from the nose, throat or skin of an attendant of the mother during labour, especially if the attendant has had a recent or current streptococcal infection. Hospital staff working in maternity units who develop a sore throat should not continue work until swabs of the throat and nose are negative for streptococci.

Other organisms that may cause puerperal sepsis originate from the mother’s faecal flora and include anaerobes, such as *Clostridium perfringens, Bacteroides* species and anaerobic cocci, and occasionally Gram-negative aerobic bacilli such as *E. coil. Staph. aureus* can cause infection also but this is unusual.

Puerperal pyrexia and constitutional upset together with possibly offensive lochia are clinical features suggestive of puerperal sepsis. In practice, puerperal pyrexia should always be investigated by collecting high vaginal swabs and, in ill patients, blood cultures arc also necessary. As soon as the cultures have been collected, blind antibiotic treatment with benzylpenicillin combined with metronidazole will usually be effective against the streptococcal and anaerobic causes of puerperal sepsis. However, penicillinase-producing strains of Gram-negative bacilli, such as ampicillin-resistant strains of Li. *co/i,* may be present and reduce the effectiveness of penicillin against streptococci. In ill patients, gentamicin could be added to the penicillin plus metronidazole blind initial therapy. A suitable alternative blind combination would be a cephalosporin plus metronidazole. Rational specific therapy can be given when the results of cultures and antibiotic sensitivities become avail able.

Post-partum pyrexia may be caused by puerperal sepsis due to the above bacteria, but *Mycoplasrna hominis* has also been demonstrated as an occasional cause. The mycoplasma organism can often be isolated from blood cultures. Tetracycline is usually clinically effective for post-partum pyrexia due to *Mycoplasma,* but in the breast-feeding mother the length of the course must be kept to the minimum.

HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND AIDS IN INFANTS

Greater than three-quarters of children so far infected with HIV acquired it either trans-placentally or perinatallv, with the remainder acquiring it through receiving contaminated blood products such as factor VIII. About 30% of infants born to HIV-infected mothers develop HJV infection, but this figure could be substantially reduced by giving zidovudine prophylaxis during pregnancy. Most infants with HIV infection develop serious clinical problems by 2-3 years and about half of infected children will have died of AIDS by 3 years of age, in the absence of specific treatment. Clinical features associated with HIV infection include recurrent bacterial infections, lymphocytic interstitial pneumonitis (sometimes associated with Epstein—Barr virus) and neurological disease, including HIV-related encephalopathy or myelitis. Microbiological investigations during the early months of life may fail to show evidence of HIV infection because there is a delay before either HIV antigen or HIV antibodies are detectable in the serum in some children and virus isolation studies are not usually practical. HIV PCR testing on the blood from the infant may be indicated to achieve an early diagnosis

In other children who stay well, a fall in maternal HIV antibodies in the infant's serum, continued health and absence of serological markers of HIV infection after 18 months of age make HIV infection an unlikely possibility.

When HIV infection is diagnosed in the mother early enough, termination of pregnancy or zidovudine prophylaxis should be considered and counselling about future pregnancies is also essential

GYNAECOLOGICAL INFECTIONS

In recent years, *'new'* infective gynaecological problems have become recognized including those associated with intrauterine contraceptive devices, vaginal tampons and gynaecological surgery.

Infections associated with intra-uterine devices

Plastic shield-type contraceptive devices that are inserted into the cervix have occasionally introduced the complication of chronic cervical and pelvic infection. Anaerobic organisms such as *Bacteroides* and anaerobic cocci are often prominent in the associated infection. Actinomycosis of the pelvic tissues has been found in a number of

patients with these devices, whereas this infection is less likely when a copper contraceptive coil is used instead of the plastic device. Removal of the infected device, culture of the device and microbiological investigation of high vaginal and cervical swabs are indicated. Metronidazole is used to treat bacteroides infection and a prolonged course of amoxycillin to treat actinomycosis, but surgery is also necessary when there is a large collection of pus or infected necrotic tissue in the pelvis, if gonococci or *Chlamydia trachomatis* are present, an attack of pelvic inflammatory disease may follow infection.

Toxic shock syndrome

In the early 1980s there were several published reports of shock associated with a febrile reaction and a rash developing in women who had used a vaginal tampon. Subsequently, it became apparent that in these women the vagina and tampon had become colonized by certain strains of *Staph. aureus* which produced toxins responsible for the clinical syndrome. In most cases, there was spontaneous cure after removal of the tampon. This disease has been reported less frequently in Britain during the last few years, but occasional cases continue to occur - not always associated with tampons

Gynaecological surgery

Surgery on the female genital tract may become complicated by wound infection, due to non-sporing anaerobes as well as aerobic organisms (*see* Chapter 10), and also by the development of a hospital-acquired urinary tract infection, especially when an indwelling catheter has been inserted.

Hospital – acquired Infection

Definition :

It is infection acquired while staying in hospital. It meets the following criteria:-

- Not found on admission.
- Temporally associated with admission or a procedure at a health care facility .
- It was not incubating at admission and may be related to a previous procedure or admission to same or other health care – facility e.g HIV acquired from previous blood transfusion .

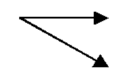
Routes of infection: -

1) Self infection (auto genous infection) .
2) Cross – infection.
3) Environmental infection

-Dust.

-Bed linen

-Air

-Moist solutions may be contaminated  Pseudomonas

Klebsiella

Factors which promote hospital acquired infection: -

1) Impaired general host defenses of the patient.
2) Impaired local host defenses of the patient .e.g injury of skin barrier.
3) Presence of hospital pathogen which are endemic.

Organisms causing Hospital acquired infection: -

In many hospital but also each hospital has specific endemic or epidemic strains of particular types of organism in certain areas .

In hospitals with large specific units e.g oncology , special care baby units opportunists organisms as well as the common pathogens are likely to cause problem

Pathogens causing hospital acquired infections include

1) Conventional pathogen e.g strepto-pyogenes .
2) Conditional pathogen e.g Bacteroides .
3) Opportunists pathogen e.g Pneumocystis carinii .

The most common organisms are staph. aureus and staph.epidermidis

-Gram Negative bacilli such as: -

E.coli is the most frequent single bacterial species associated with hospital acquired infection .

Klebsiella , *Proteus* species and *Pseudomonas* are also common cause .

- Fungal and viral infection are only occasionally acquired in hospital .
- Protozoa are rare.

Laboratory diagnosis Of Hospital – acquired Infections :

-Samples collected from the patient includes Blood culture, sputum, tracheostomy wound swabs and other samples. Perform cultured before antibiotics therapies and judged accurately if the isolate is just contamination or already a true pathogen.

-Samples from the environment include all equipments in the ICU and from antiseptic solutions according to the place of environmental assay.

-Typing of the isolated organisms from both patients, environment and personnel by the following typing methods:

1) Bio typing
2) Sero typing
3) Phage typing
4) Bacteriocine typing
5) DNA finger printing
4)

Frequency of Nosocomial Infection

- Nosocomial infections occur worldwide.
- The incidence is about 5-8% of hospitalized patients, 1/3 of which is preventable.
- The highest frequencies are in East Mediterranean and South-East Asia.
- A high frequency of N.I. is evidence of poor quality health service delivered.

Impact of Nosocomial Infections

- They lead to functional disability and emotional stress to the patient.
- They lead to disabling conditions that reduce the quality of life.
- They are one of the leading causes of death.
- The increased economic costs are high: Increased length of hospital stay (SSI - 8.2 days), extra investigations, extra use of drugs and extra health care by doctors and nurses.

Organisms causing N.I. can be transmitted to the community through discharged patients, staff and visitors. If organisms are multi-resistant they may cause significant disease in the community.

Nosocomial Infections Cost

➲ The cost varies according to the type and severity of these infections.

➲ An estimated 1 to 4 extra days for a urinary tract infection, 7 – 8 days for a surgical site infection, 7 – 21 days for a blood stream infection, and 7 – 30 days for pneumonia.

➲ The CDC has recently reported that US$5 billion are added to US health costs every year as a result of NI.

➲ In Egypt one LE spent for infection control saves LE 60 spent on NI.

Nosocomial Infection Sites

✦ Urinary tract infection: most common type of N I (30-40% of reported cases), associated with an indwelling urinary catheter or instrumentation.

✦ Lower respiratory and surgical wound infections are the next (each about 15%).

✦ Less frequent include bacteraemia (5%), intravenous site infection, gastrointestinal tract and skin infections.

Criteria of Nosocomial Infections

Surgical site infection	Any purulent discharge, abscess or spreading cellulitis at the surgical site during the month after operation
Urinary infection	Positive urine culture (1 or 2 species) with at least 100000 bacteria/ml, with or without clinical symptoms
Respiratory infection	Respiratory symptoms with at least 2 signs: cough; purulent sputum; new infiltrate on chest, appearing during hospitalization
Vascular catheter infection	Inflammation, lymphangitis or purulent discharge at the insertion site
Septicaemia	Fever or rigours and at least one positive blood culture

Factors Influencing N.I

- The microbial agent
- Patient susceptibility
- Environmental factors

Microbial Agent

- Many sick people are treated in a closed area; micro-organisms, frequent contact between carriers & susceptible, contaminated waste, equipment and supplies to be handled.
- Developing of clinical disease depends on organism s virulence, infective dose and patient resistance
- Bacteria are the most common pathogens.

1. *Commensal bacteria:* found in normal flora of healthy humans, prevent pathogenic bacterial colonization eg skin, colon, vagina
2. *Pathogenic bacteria:* have great virulence and cause infection as :

- Anaerobic gram +ve rods e.g Clostridium causing gangrene.
- Gram +ve bacteria: Staph. aureus found on skin &nose. - Beta - hemolytic Strep.
- Gram -ve bacteria as E.coli, Proteus, Klebsiella.
- legionella species.

- *Viruses:* HIV, HBV, HCV can be also be transmitted through blood & B F (transfusion, injections, dialysis)
  respiratory syncytial virus, rota virus, ebola, infleunza, herpes simplex viruses.
- *Parasites & Fungi:* e.g. Giardia lamblia is easily transmitted between adults or children, Aspergillus sp. affecting imunocompromised.
- Scabies an ectoparasite causing outbreak.

Patient Susceptibility

- ***Age:*** infants and old age have decreased resistance to infection.
- ***Immune status:*** Patients with chronic diseases as malignancy, leukaemia, diabetes mellitus, renal failure or AIDS have increased susceptibility to infection.
- *Immunosuppressive drugs or irradiation*

Environmental Factors

- Healthcare settings are environment where both infected persons and persons at high risk of infection congregate.
- Crowded conditions within hospital, frequent transfers of patients between units.

➢ Microbial flora may contaminate objects, devices and materials which subsequently contact susceptible body sites of patients.
Transmission

- **Where do nosocomial infection come from?**

✓ ***Endogenous infection:*** When normal patient flora change to pathogenic bacteria because of change of normal habitat, damage of skin and inappropriate antibiotic use. About 50% of N.I. Are caused by this way.

✓ ***Exogenous cross-infection:*** Mainly through hands of healthcare workers, visitors, patients.

✓ ***Exogenous environmental infections:*** several types of micro-organisms survive well in the hospital environment (hospital flora):

* In water, damp areas and occasionally in sterile products or disinfectants eg pseudomonas,
  Acinetobacter, Mycobacterium.
* On items such as linen, equipment and supplies
* In food.
* In fine dust and droplet nuclei

✓ ***Some procedures that save life*** may increase risk of infection e.g urinary catheters, I.V.L inhalation therapy, surgery.

✓ ***Inappropriate use of antibiotics***

Basics of Infection Control

☑ Prevention of nosocomial infection is the responsibility of all individuals and services provided by healthcare setting.

☑ To practice good asepsis, one should always know: what is dirty, what is clean, what is sterile and keep them separate.

☑ Hospital policies & procedures are applied to prevent spread of infection in hospital.

Infection Control Program

- A comprehensive, effective and supported program is essential for reducing infection risk and increasing hospital safety.

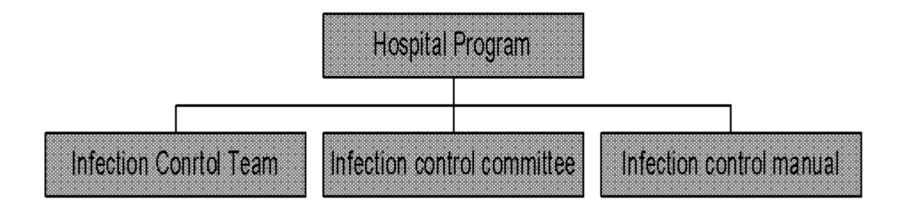
- It should include surveillance, preventive activities and staff training.

I. National program developed by Ministry of Health: to support hospital programs. It sets national objectives, develops and updates guidelines recommended for health care.

II. Hospital programs including:

1) major preventive efforts; keeping in mind
   patients and staff.
2) It must be supported by senior management and

provided with sufficient resources.
3) It must develop a yearly work plan to assess and promote all good health care activities



Infection Control Team

- The optimal structure varies with hospitals types, needs and resources.
- Hospital can appoint epidemiologist or infectious disease specialist, microbiologist to work as infection control physician.
- Infection control nurse who is interested and has experience in infection control issues.
- Team should have authority to manage an effective control program.
- Team should have a direct reporting with senior administration.
- Infection control team members or are responsible for day-to-day functions of IC and preparing the yearly work plan.
- They should be expert and creative in their job.

Infection Control Committee

- It is a multidisciplinary committee responsible for monitoring program policies implementation and recommend corrective actions.

- It includes representatives from different concerned hospital departments & management. They meet bimonthly.

- It establishes standards for patient care, it reviews and assesses IC reports and identifies areas of intervention.

Infection Control Manual

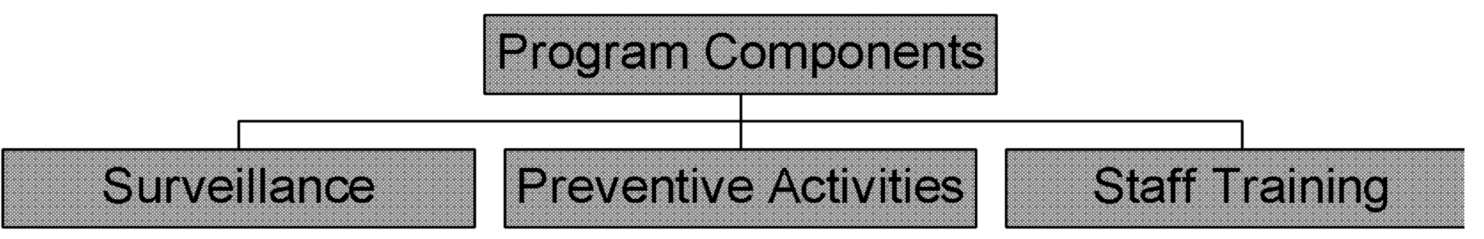
- Every Hospital should have a nosocomial infection prevention manual compiling recommended instructions and practices for patient care.
- This manual should be developed and updated in a timely manner by the infection control team.

☐ It is to be reviewed and accepted by infection control committee.

Infection Control Responsibility

✯ Role of every hospital department and service units must be identified, documented as manuals kept in accessible place.

✯ Job description of every hospital staff; defining details of his duties must be discussed before employment. Infection control precautions should be part of the routine work and stressed for that.



NOSOCOMIA INFECTION SURVEILLANCE

- Nosocomial infection rate in a hospital is an indicator of quality and safety of care.
- Surveillance to monitor this rate is essential to identify problems and evaluate control activities
- The ultimate aim is the reduction of infection rate and their costs.
- The term surveillance implies that observational data are regularly analyzed.

Key points in Surveillance

- Active surveillance (Prevalence and incidence studies)
- Targeted surveillance (site, unit, priority-oriented)
- Appropriately trained investigators
- Standardized methodology
- Risk- adjusted rates for comparisons

Organization for surveillance

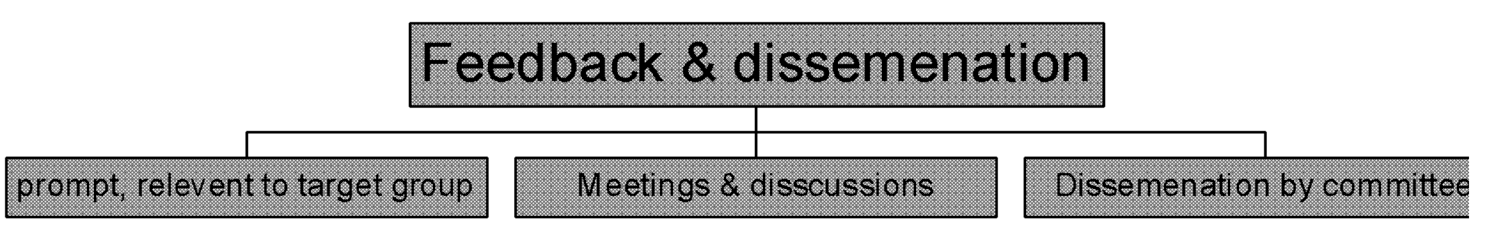
Data collection and analysis

Ward activity
devices or procedures
fever & inf. signs
antibiotics & charts

Laboratory reports
culture& sensitivity
resistance patterns
serologic tests

Data elements &analysis
patient data & infection
population & risks
computerization of data

Organization for surveillance



Scope of Infection Control

Aiming at preventing spread of infection:

Standard precautions: these measures must be applied during every patient care, during exposure to any potentially infected material or body fluids as blood and others.

Components:

A. Hand washing.
B. Barrier precautions.
C. Sharp disposal.
D. Handling of contaminated material.

A.HAND WASHING

+ Hand washing is the single most effective precaution for prevention of infection transmission between patients and staff.
+ Hand washing with plain soap is mechanical removal of soil and transient bacteria (for 10- 15 sec.)
+ Hand antisepsis is removal & destroy of transient flora using anti-microbial soap or alcohol based hand rub (for 60 sec.)
+ ***Surgical hand scrub:*** removal or destruction of transient flora and reduction of resident flora using anti-microbial soap or alcohol based detergent with effective rubbing (for least 2-3 min)

\+ ***Our hands and fingers*** are our best friends but still could be our enemies if they carry infective organisms and transmit them to our bodies and to those whom we care for.

\+ ***Sinks & soap*** must be found in every patient care room. Doctors, nurses must comply to hand washing policy.

When to Wash our Hands

1. Before & after an aseptic technique or invasive procedure.
2. Before & after contact with a patient or caring of a wound or IV line.
3. After contact with body fluids & excreta removal.
4. After handling of contaminated equipment or laundry.
5. Before the administration of medicines
6. After cleaning of spillage.
7. After using the toilet.
8. Before having meals.
9. At the beginning and end of duty.
10. Gloves cannot substitute hand washing which must be done before putting on gloves and after their removal.

How to Wash our Hands

â Jewelry must be removed. If unable to remove rings, wash and dry thoroughly around them.

â Wet your hands with running warm water, dispense about 5 ml of liquid soap or disinfectant into the palm of the hand.

â Rub hands together vigorously to lather all surfaces and wrist paying particular attention to thumbs, finger tips and webs.

â Rinse hands thoroughly.

â Turn off water using elbow-on elbow taps, dry hands thoroughly on a paper towel OR where elbow taps are not present, first dry hands, thoroughly, then turns off the taps using fresh paper towel.

â Hand cream can be used on persona basis.

â If a staff member develops a skin problem, he or she must consult dermatologist.

B. Barrier Precautions

1. ***<u>Gloves:</u>***

Disposable gloves must be worn when:

a) Direct contact with B/BF is expected.
b) Examining a lacerated or non-intact skin e.g wound dressing.
c) Examination of oropharynx, GIT, UIT and dental procedures.
d) Working directly with contaminated instruments or equipment.
e) HCW has skin cuts, lesions and dermatitis

å *Sterile gloves are used for invasive procedures.*

å GLOVES MUST BE of good quality, suitable size and material. Never reused. ***2) Masks & Protective eye wear:***

å

å MUST BE USED WHEN: engaged in procedures likely to generate droplets of B/BF or bone chips.

å

å During surgical operations to protect wound from staff breathings, …

å

å Masks must be of good quality, properly fixed on mouth and nasal openings.

å

3) Gowns/ Aprons:

Are required when:

- Spraying or spattering of blood or body fluids is anticipated e.g surgical procedures.

- Gowns must not permit blood or body fluids to pass through.

- Sterile linen or disposable ones are used for sterile procedures.

C.Sharp precautions

- Needle stick and sharp injuries carry the risk of blood born infection e.g AIDS, HCV,HBV and others.
- Sharp injuries must be reported and notified
- NEVER TO RECAP NEEDLES
- Dispose of used needles and small sharps immediately in puncture resistant boxes (sharp boxes).
- Sharp boxes: must be easily accessible, must not be overfilled, labeled or color coded.
- Needle incinerators can be another safe way of disposal.
- Reusable sharps must be handled with care avoiding direct handling during processing

D. Handling of Contaminated Material

1. *Cleaning of B/BF spills:*
   a- wear gloves.
   b- wipe-up the spill with paper or towel.
   c- apply disinfectant.
2. *Cleaning & decontamination of equipment:*
   protective barriers must be worn.
3. *Handling & processing lab specimens:*

must be in strong plastic bags with biohazard label

4. Handling and processing linen:

Soiled linen must be handled with barrier precautions, sent to laundry in coded bags.

5. Handling and processing infectious waste:

a. must be placed in color coded, leakage proof bags, collected with barrier precautions

b. contaminated waste incinerated or better autoclaved prior to disposal in a landfill.

Environmental control:

1. Including physical facility plans must meet quality and infection control measures. Patient equipment positioning and installation, traffic flow.
2. Cleaning of hospital environment and dis-infection according to policies.
3. Proper air ventilation.
4. Water pipes examination, check its quality.
5. Proper waste collection and disposal.
6. Cleaning and dis-infection of equipment.
7. Proper linen collection, cleaning, distribution
8. Food : ensure quality and safety.
9. Sterilization:

Central sterilization department serving all hospital departments compiling with infection control precautions.

Patient protection :

corrective measures before major procedure, vaccination, proper use of antibiotics.

* Isolation precautions.
* Limiting endogenous risk

Staff health promotion and education:

1. HCW are at risk of acquiring infection, they can also transmit infection to patients and other employee.
2. Employee health history must be reviewed, immunizations recommendations to be considered.
3. Release from work if sick, occupation injury must be notified.
4. Continuous education to improve practice, better performance of new techniques.

1- Hospital – acquired urinary tract infections

- The most common type of hospital infection (about 25%)
- The source auto- genous from patient's own faecal flora or from the environment (urinals , wash bowl) .

- Colonization of the patient's large intestine by hospital resistant strains of Gram – negative bacilli occurs when the patient stays in hospital more than few days or when patient take antibiotics .

- E.coli is the most common pathogen especially resistant strains to sulfonamides and ampicillin other pathogens such as klebsiella aerogenes, Enterococcus spp. (Vancomycin resistant) and Pseudomonas are common .

Occasionally candida and Serratia marscenes may cause such infection . Patients with impaired immune system may become infected wit T.B and Nocardia asteroids , salmonella spp. Or Papoviruses .

- Mixed infections are common with catheterization

Effects of UII : -

1) Asymptomatic infection .
2) Symptomatic infection
3) Prolonged hospital stay .
4) Pyelonephritis , hemorrhage after operation
5) Septicemia shock .

Catheterization hazards : -

Patients with catheter have the following dangers:

- 10-30% develop UTI by the fifth day .
- 100% develop UTI by 2-3 weeks .

Prevention of catheter – associated urinary infection : -

- Good aseptic technique to the surrounding area with chlorhxidine solution and cream .
- Never touch the tip of the catheter .
- Wash hands with chlorhexidine solution .
- Chlorhexidine installation into the bladder .
- Early removal of the catheter .
- Change it within few days or when dirty .

-Take care not to soil the hands of the staff .

4) Policies for control of hospital acquired urinary tract infections :

This depends upon the extent to which these policies are effectively carried from day to day .

- Isolate patients with multiple antibiotic resistant strains .
- Sponge with antiseptic solution should be put between catheter and urethra especially in females.
- Repeated self catheterization with use of antiseptic solution is preferred in paraplegic patient .

2-Surgical wound Infection

1) The type of the wound is the most single factor associated with the development of wound infection . The major types of surgical wounds can be classified as follows : -

-Clean operation wounds in area not involve regions of gastrointestinal tract, respiratory tract or genitourinary tract. It is associated with very low rates of infection 2-5%.
- Contaminated operation Surgery that involves a site with known normal flora (apart from skin) e.g. operation on colon , gall bladder , mouth or vagina .
- Infected operation wounds , the operation site may be infected at time of surgery e.g. incision of an abscess .
2) Surgical team:
- Skill of surgeon
- Good aseptic techniques
- Carriage of staph. aureus .
3) Age and general condition of the patient .
4) Persistence of local structural abnormal .
5) Ward factors post operatively
Complication of wound Infections : -
- Delayed wound healing
- Failure of graft .
- Infections of bones , joints , peritoneal cavity .
- Septicemia
Types of hospital acquired wound infections include : -
- Ward infection
- Theatre infection
(How to differentiate ?)
Prevention of theatre infection : -
- Protective of theatre clothing .
- Gloves for the hands after chlorhexidine antiseptic solution
- Movements of staff should be reduced to a minimum .
- Very clean theatre with good managing of the air direction around the operative table .
- Disinfect the skin of the patient and many use preoperative baths with hexachlorophene or chlorhexidine detergents .
Prevention of wards infections : -
- Isolation rooms for wards should be available for patients infected with MRSA or with severe wound infections especially in high risk surgery e.g. cardio thoracic orthopedic , neuro surgical units and ICU .
- Adequate non touch technique for dressing of the wound .
- Suitable bad spacing between patients (2.5m)to decrease air or dust spread .
- The patient should be admitted for the shortest time before the operation to decrease the chances of colonization or infection with hospital strains .
- Restrict the use of prophylactic antibiotics .

- Exclude patients with skin disease from the word .
- Exclude members of staff with boils , abscess or other infected skin lesions .

If an out breaks occur , how to control ?

- Be sure that it is cause by the same epidemic strain with similar antibiotic resistance pattern and phage type .
- Isolate the patient .
- The medical staff shown to be carrier should temporarily cease the work and use chlorhexidine on the affected area with all hygiene measure .
- Close the ward in severe cases .
- Use vancomycin for therapy .

3-Acute Lower Respiratory tract Infection

- Third most common infection in hospital acquired infection
- Patients predisposed are usually pediatric patients or elderly patients with predisposing chest conditions .
- Causative pathogens include , strepto . pneumoniae , staph. aureus , influenza A or B , respiratory syncytial virus and legionella species.

Infections in ICU

- It is very common to find high rates of infections in intensive care units.
- Samples collected from the patient includes Blood culture , sputum, tracheostomy wound swabs and other samples. Perform cultured before antibiotics therapies and judged accurately if the isolate is just contamination or already a true pathogen .
- Samples from the environment includes all equipments in the ICU and from antiseptic solutions .
- Preventive measures are the same as before and the measures for the equipments include :

• Adequate cleaning of the equipment in between patients
• Heating water in the ventilator to 50°C every day .
• Use of autoclavable ventilator or decontaminate it with ethylene or formaldehyde gas .
• Anesthetic bags , suction apparatus , face masks may be efficiently cleaned with washing machine or by low temperature disinfectant .

Infective hazards of intravenous fluid

- Between 0.2 – 8% of patients receiving intravenous fluid develop septicemia and may develop endotoxic shock if gram – negative bacilli multiply at the additive solution added to the fluid .

Causative organisms : -

• Gram negative bacilli contaminating fluid solutions .

- Bacterial or viral contamination of blood and blood products including HIV , HTLVI , hepatitis viruses , Epestein Barr virus , CMV , Treponema pallidam and malarial parasites .

Prevention of infections with Fluid Infusion.

- Perfect antiseptic technique to the skin over the area used with shaving of the excess hair and palpate the vein before use of antiseptic solution (chlorohexidine or iodine)
- Place the needle in the place and needs to be anchored securely as excessive movement predisposes to site infection
- Topical Betadine antiseptic solution and if needed antifungal cream may be added .
- Inspect the site of the drip , if infected with draw the complete set immediately and insert it at different site .
- Change the complete set every 24-48h
- Restrict the use of antibiotic therapy

Diagnosis of infections associated with intravenous infusion therapy : -

- With draw blood samples for blood culture .
- Send the bottle , cannula and there catheter to the microbiological lab.
- Inspect the bottle for presence of defects , and with draw fluid for microscopic examination , culture at 35°C , 4°C and at room temperature .
- Culture the catheter tip or cannula on broth or by rolling moistened cannula on surface of blood agar plate – organisms isolated include staph. epidermidis , staph. aureus , candida albicans , klebsiella species , serratia , pseudomonas aeruginosa .
- Begins blind therapy with cloxacillin plus gentamicin .

Policies for prevention of infection to or / and from Health works

1) **Tuberculosis** : -

- Staff are Mantoux tested and have chest X ray at start of hospital employment
- Mantoux negative BCG immunization and be sure to convert mantoux negative to positive .
- Mantoux positive Deals with patients with open T.B or samples known to have T.B. bacilli .
- Isolate patient with open T.B in single room until 2-3 weeks after antituberculous therapy .
- Each sputum sample should be handled as if it had T.B and never be opened in the ward .
- In the lab See precautions of handling sputum samples .

2) Viral Hepatitis :

- Exclude staff with HBsAg positive from renal dialysis units or from carrying exposure – prone procedures

- Precautions should be taken in the lab. To prevent infections to workers .
- All staff with regular contact with blood should have anti HBS with titer > (10 lu/L)

Precautions to prevent viral hepatitis : -

- Isolate the patients (stool / urine / needle isolation)
- Take blood samples with gloves
- Transport to the lab in sealed plastic bags.
- Samples are put in upright position in non leaking screw caps containers
- Samples and requests should be labeled as hepatitis risk.
- Take care of samples from suspected patients.
- Accidental prick to staff should be reported to senior staff, take hyper immune globulin with 24-48 hours and vaccinate if HBS antibodies is negative.
- Wearing of two gloves , waterproof apron and goggles together with many other precautions are necessary when surgery is carried out on patients with hepatitis B. (universal precautions) .
- Staff members positive for HBsAg or e antigen should not work in dialysis unit , oncology , surgery or transplant unit .

3)AIDS :

- Blood and blood products should be screened for HIV 1/2.
- Universal precautions against blood – borne viruses should be taken when blood or blood staining splashing are anticipated as risk .
- Post exposure drug prophylaxis is recommended for staff sustaining a sharp injury from a patient or HIV .

Policies for Control of Hospital –acquired Infections

Each hospital has many procedures and policies which attempt to reduce the chances of hospital infection occurring , but the extent to which these policies are effectively carried out on a day to a day basis varies greatly between different hospitals or different areas in the hospital .

Procedures for Control Include: -

- Sterilization and disinfection of contaminated items .
- Disposal of infected rubbish or linen .
- Aseptic techniques in the operating theatre
- Procedures carried in wards such as changing of wound dressings , urinary catheterization and setting up of an intravenous drip .
- Protective isolation of infected patients or protective isolation of highly susceptible patients .
- Use of antibiotics therapy according to a greed policy .
- Education of hospital staff in hospital hygiene .
- Good staff health facilities .

- Adequate use of the clinical microbiology for the precise bacteriological diagnosis .
- Control infection committee which helps to design hospital policies and discusses any difficulties encountered during the implementation of these policies .

Opportunistic infections

Many opportunistic infections are acquired in hospital. Opportunistic infections are a major cause of illness and death in oncology patients and the leading cause of death in recipients of renal transplants. Severely immunocompromised patients may develop simultaneous infections with several different types of 'opportunist' organisms. Opportunistic infections are the usual cause of death in patients with the acquired immunodeliciency syndrome (AIDS).

'OPPORTUNIST' Organisms

The term 'opportunist' is not an exact one. 'Opportunist' organisms have three main characteristics:

1. They are usually organisms of low pathogenicity, e.g.

- Pseudomonas aeruginosa
- Staph. Epidermidis
- Candida albicans
- Cytomegalovirus
- *Pneumocysus carinii*

2. They cause serious infections mainly when the host's defense mechanisms against infection are impaired, e.g.

• In patients receiving treatment for acute leukaemia or lymphoma, recipients of renal or other transplants who are immunosuppressed, and patients with AIDS

3. They can behave as conventional pathogens' but under opportunistic conditions may cause atypical clinical presentations or disseminated lesions, e.g.

Mycobacterium tuberculosis causing a PUO illness in immunosuppressed patients due to miliary TB or *Strongyloides stercoralis* causing overwhelming life-threatening infection in a persistently immunosuppressed patient

Examples of opportunist organisms are included in *Table 7. 1* together with brief notes on their associated infections and the specimens required for microbiological diagnosis.

'Opportunist' bacteria

Gram-negative bacilli

Gram-negative bacilli are the most common 'opportunist' pathogens and their sources are either

Endogenous from the patient's alimentary tract flora, the most frequent source, causing 'autogenous' infections ('self-infections')

or Exogenous from infected or colonized lesions from other patients or from moist contaminated sites in the hospital environment Cross-infection may be associated with the spread of Gram-negative bacilli by the hands of hospital staff

Table 7.1 Some opportunistic infections and specimens for diagnosis

Organism	Opportumistic infection	Specimens for diagnosis
Bacterial :		
- Psedomonas aeruginosa Klebsiella aerogenes Serratia marcescens Other "Coliforms" and pseudomonas species	1.Gram-negative pneumonia 2.Gram-negative septicaemia (following invasion of blood by organisms, after localized infections, or from normal gut flora)	Blood cultures and sputum Blood cultures and specimens from infected sites including urine and sputum
- Legionella pneumophila	Legionnaires' disease	Bronchial washings, sputum and blood cultures for culture for Legionella paired sera for antibodies, urine for antigen
- Staph. aureus	Severe staphylococcal pneumonia, septicaemia or persistent abscesses	Blood cultures, sputum and pus for culture
- Nocardia asteroids	Nocardiosis, especially involving lungs, subcutaneous tissues or kidneys	Sputum, pus from empyema or skin abscess, urine for microscopy and culture
- Listeria monocytogenes	Listeriosis, involving lungs, blood or CNS	Blood cultures and CSF
- Mycobacterium tuberculosis and BCG	Miliary tuberculosis Disseminated and pulmonary BCG	Sputum, EMSUs, bone marrow and liver biopsy for microscopy and culture of acid-fast bacilli
- Mycobacterium avium-intracellulare	Disseminated infection in patients with AIDS	Faeces, bone marrow and blood cultures and/or microscopy for acid-fast bacilli
Viral:		
Herpes viruses 1. Herpes simplex	a. Severe or generalized herpetic infection of skin and mucous membranes b. Herpetic eye infection-danger of corneal dendritic ulcers c. Herpes simplex pneumonitis, hepatitis or encephalitis	Vesicular fluid and swabs of lesions into viral transport medium for culture; rapid examination of fluid or tissue by electron microscopy or fluorescent antibody method
2. Cytomegalovirus (CMV)	a. Disseminated cytomegalovirus inclusion disease and PUO b. CMV pneumonia c. CMV hepatitis	Throat swab and urine for viral culture; liver biopsy and other tissue for microscopic Examination for CMV inclusions; paired sera for antibodies, whole blood for quantitative CMV PCR
3. Varicella-zoster Virus (VZ)	a. Severe or disseminated zoster (shingles) infection-may be rapidly fatal b. Varicella pneumonitis	Vesicle fluid for electron microscopy (culture not usually feasible) Paired sera for VZ CSFs

Measles virus	Giant cell pneumonia, especially in children who have not previously had measles or who were not previously immunized when their host defences were apparently normal- carries high mortality rate. Especially dangerous in leukaemia children	Throat swab for virus culture; paired serafor measles CSFs
Hepatitis B virus	Symptomless carriage frequent in immunosuppressed and uraemic patients-high titres of hepatitis virus may be present – risks to attendant staff as well as other patients	Serum for HBsAg and other hepatitis serological tests (see chapter 16)
Fungal:		
Yeasts Candida aqlbicans and other Candida spp.	Systemic candidiasis-broncho-pulmonary oesophageal, peritoneal or renal lesions; septicaemia and endocarditis	Blood culture and culture and microscopy on specimens from relevant sites
Cryptococcus neoformans	Cryptococcosis – chronic granuloma in lungs or CNS – and disseminated infection in patients with AIDS	Sputum, CSF for microscopy and culture; blood cultures; tests for cryptococcal antigen in CSF
Filamentous fungi :		
Aspergillus fumigatus and other Aspergillus spp.	Pulmonary or disseminated Aspergillus . In the latter form lesions may occur in lungs, brain and liver. Rarely aspergillus endocarditis may be the main lesion	Sputum for microscopy and culture; blood cultures; biopsy material for microscopy and culture; serology – aspergillus precipitins (may be negative)
Mucor spp.	Mucormycosis	Biopsy material for microscopy and culture
Dimorphic fungi:		
Histoplasma capsulatum	Histoplasmosis – pulmonary and disseminated histoplasmosis – mainly affecting patients who have stayed in N. America or W. Indies	Sputum, bone marrow aspirate, liver biopsy for microscopy and culture; blood cultures; serum for histoplasma CSF may be negative)
Protozoal :		
Pneumocystis carinii (now a 'funfus')	Pneumocystis interstitial pneumonia	Induced sputum, bronchial washings and lung biopsy – immunofluorescent or silver stain microscopy
Toxoplasma gondii	Toxoplasmosis – severe and disseminated lesions may occur affecting brain and myocardium	Biopsy material of lymph nodes or other tissues for histology and paired sera for toxoplasma dye or fluorescent antibody tests
Cryptoporidium	Cryptosporidial diarrhea	Faeces– acid – fast

	in patients with AIDS	microscopy to see characteristic ova
Babesia spp.	Babesiosis – fever, anaemia and myalgia in splenectomized patient (possible following tick bite)	Thick and thin blood films for Giemsa staining – parasite seen in red cells
Worms : Stronglyloides stercoratis	Disseminated strongyloidiasis – affecting organs following invasion of blood from the gut – may be fatal and occur many years after the original infestation (eosinophilia may be absent)	Faeces, jejunal biopsy, sputum or gastric aspirate for microscopy for the larvae

AIDS, acquired immunodeficiency syndrome; BCG, Bacille Calmette – Guerin; CFT, complement fixation test; CMV, cytomegalovirus; CNS, central nervous system; CSF, cerebrospinal fluid; EMSU, early morning specimen of urine ; PCR, polymerase chain reaction .

Examples include E. coli, Klebsiella aerogenes, Proteus mirabilis, Pseudornonas aeruginosa, P. cepacia and other Pseudomonas species, Enterobacter, Acinetobacter, and non-pigmented strains of Serratia marcescens. All of these organisms have been increasingly isolated from blood cultures during, the last 30 years and this is largely due to the increased incidence of 'opportunistic' conditions .
There is a high incidence of opportunistic Gram-negative infections in special units such as oncology units, intensive care and special care baby units, neurosurgical units, liver units, renal units and burns units.
Factors that predispose to Gram-negative infections in these units include instrumentation and the frequent administration of antibiotics. Many of the Gram-negative bacilli are multiple antibiotic resistant and this resistance is often R factor (plasmid) mediated; there may also be 'cross-infection' with plasmids mediating 'en bloc' multiple antibiotic resistance between different strains of the same species or between strains of different Gram-negative bacterial species. The epidemiology of infections occurring in intensive care units is typical of that seen in special units.
Gram-positive bacteria
These are important causes of opportunistic infections although less frequent causes than Gram-negative bacilli. Examples include *Strep. pneumeniae* in splenectomized children (sometimes causing serious and recurrent infections), *Listeria monocytogenes* in lymphoma patients, *Staph. aureus* in neutropenic patients (causing pneumonia and septicaemia) and *Staph. aureus* or *Nocardia* asterozdes in children with chronic granulomatous disease. *Strep. agalactiae* (Lancefield group B haemolytic streptococcus) may cause serious infections in low birth

weight neonates and may also rarely cause endocarditis in elderly debilitated patients. *Staph. epidermidis* is an increasingly frequent opportunist organism in immunocompromised or debilitated patients who have either an intravenous infusion or a catheterized urinary tract or continuous ambulatory peritoneal dialysis (CAPD). This staphylococcus is also an important cause of infection in patients with a Spitz-Holter valve, a prosthetic heart valve or a hip joint prosthesis. Viridans streptococci have also recently been noted to cause septicaemia occasionally in neutropen ic immunosuppressed patients.

Acid-fast bacilli

Mycobacterium tuberculosis in an important 'opportunist' causing disseminated lesions in renal transplant and other patients receiving prolonged immunosuppressive drugs and in patients with lymphoma. BCG and some 'opportunist' ('anonymous') mycobacteria have also caused infections in immunocompromised patients, although these are very rare compared with NI. tuberculosis, for example disseminated life-threatening infection due to BCG in a child with chronic granulomatous disease.

Mycobacteriuni avium-intracellulare frequently causes disseminated infection in patients with AIDS.

Opportunist fungi

Fungal causes of opportunistic infection are rare compared to bacterial causes, but are important causes of life-threatening infections in persistently immunocompromised patients. Examples include Candida albicans and other *Candida* species, *Pneumocyssis* carinii, *Aspergillus funugatus* and other *Aspergillus* species, *Cryptococcusus neoformans, Mucor, Histoplasma capsulatum* and *Coccidioides immitis.* The latter two fungi are mainly relevant in patients who have, at some time, stayed in the endemic regions of North America.

These infections particularly affect patients with decreased cellular immunity or neutropenia, including those with lvmphomas or sarcoidosis and recipients of renal transplants. Previous broad-spectrum antibiotic therapy is an important predisposing factor, especially for *Candida* and *Aspergiilus* infections. Serious Candida lesions may develop in pharyngeal— oesophageal, bronchial, renal and endocardial sites, sometimes in association with candidnemia. Patients who are having peritoneal dialysis can occasionally develop a candida peritonitis. Aspergillus infection mainly involves the lungs but subsequent dissemination can involve one or more other sites including the brain, pituitary, kidneys and heart. Not only may multiple sites become infected by one fungus, but simultaneous infection due to more than one type of fungus may also occur. The mortality rate of opportunistic fungal

infections is high, especially in aspergillus infections in immunosuppressed patients.

Viral, protozoal and helminth opportunist organisms

Viruses, protozoa and helminths may cause life-threatening infections in patients with impaired cellular immunity including patients with lymphoma, immunosuppressed patients and patients with AIDS *(see Tables)*.

Table 7.2. Impaired host defences and associated opportunistic infections

	Bacterial			**Viral**	**Fungal**			**Protozoal**
	Gram – negative bacilli, e.g. E. coli, Klebsiella, pseudomonas, Serratia	*Gram –positive cocci, e.g. staph. Aureus, strep. Pneumoniae*	*'Intraccllular' e.g. Listeria, Legionella, Myco. Tuberculosis*	*Herpes simplex, CMV, varicella-zoster, measles*	*Candida albicans*	*Cryptococcus neoformans*	*Aspergillus fumigatus*	*Pneumocystis Carinii*
Neutropenia e.g. during treatment of acute leukaemia	+++	+++	+	+	+++	+	+++	+
Cellular immunity deficient e.g. in patients with AIDS lymphomas, acute lymphoblastic leukaemia, sarcoidosis, or recipients of transplants treated with prolonged immunosuppression	+	+	+++	+++	++	+++	++	+++
Humoral immunity deficient e.g. post-splenectomy* congenital immunoglobulin deficiency	+	+++	+	+	+	–	–	+

+++ Strong association .

++ Moderate association .

- Splenectomy patients predisposed to serious infection with capsulated organisms including pneumococci and haemophilus .

Causative organism	Usual sources	Means of spread to other patients include
Bacterial examples Mycobacterium tuberculosis	Usually reactivation of past tuberculous focus; occasionally follows contact	Inhalation of acid – fast bacilli from contact with open pulmonary TB
Legionella pneumophila	Contaminated water in air conditioning plants, humidifiers, shower equipment	Inhalation of aerosols containing Legionella from an environmental source (no case – to – case spread)
Neocardia asteroides	Contaminated air and dust; sputum from infected patient	Air and dust
Viral examples Cytomegalovirus Herpes simplex Viricella – zoster (VZ) virus	Usually reactivation of latent infection; VZ infection occasionally follows contact with a case of shingles or chickenpox	Spread of herpes simplex or cytomegalovirus possible staff – probably unusual; spread of varicella – zoster by contact via infected respiratory droplets or vesicle material is likely to susceptible staff or patients (strict isolation and contact only with VZ – immune staff is necessary)
Meastes virus	Respiratory droplets from another case	Inhalation of infected respiratory droplets
Fungal examples Aspergillus fumigatus Cryptococcus neoformans	Contaminated air and dust	Air and dust (case – to – case spread is probably rare)
Pneumocystis carimi	Unknown	Outbreaks may occur-epidemiology not known
Hebminth example Strongyloides stercoralis	Dissemination to lungs and other sites from previous intestinal strongyloidiasis	(No case – to – case spread in hospital)

OPPORTUNISTIC CONDITIONS

The ‘opportunistic conditions’ present in an individual patient greatly influence the type of infections that the patient may develop. These conditions include:

Impaired host defenses

This is the most important group of 'opportunistic conditions'. The three main types of impaired general host defenses include severe neutropenia (granulocytopenia), impaired cellular immunity and impaired humoral immunity *(see Table 7.2)*.

Severe neutropenia is probably the most frequent type of impaired host defense mechanism encountered in patients who develop hospital acquired (nosocomial) infection and it may result in serious infections. Infections in neutropenic patients are most often bacterial and death often results from either pneumonia or septicaemia. The chances of serious bacterial infections occurring is inversely related to the granulocyte count, when the granulocyte counts are less than 500-1000 per mm^3. The risk of infection is also closely related to the duration of severe neutropenia. Virtually all patients tend to develop serious bacterial infections when the blood granulocyte count has remained less than 300 per mm^3 for more than 2 or 3 weeks.

Patients with impaired cellular immunity may suffer from 'intracellular', bacterial, viral, fungal, protozoal or helminth infections and simultaneous infection with different organisms is frequent. AIDS is now the most common cause of serious impairment of cellular immunity, in the community, in many countries. An impaired humoral immune response is particularly associated with serious bacterial infections. Severe impairment of an immunoglobulin response to infection is less frequent in adults than an impaired cellular immune response.

Impaired immunity and disorders affecting phagocytes may be due either to primary (congenital) or secondary (acquired) causes. Congenital immunodeficiency is discussed in is much less frequent than impaired host defenses due to acquired causes. Secondary causes include:

a. Extremes of age

e.g. low birth weight neonates and the elderly

b. Acquired diseases including

i. *Acquired immunodeficiency syndrome (see* Chapter 22)

ii. *Diseases of the reticuloendothelial system* e.g. leukemia, lymphoma, myeloma

iii. Immunoglobulin deficiency associated with disease

e.g. nephrotic syndrome, protein-losing enteropathy, severe malabsorption syndrome

iv. *Metabolic disease*

e.g. diabetes n1ellitus, uremia, liver failure

v. *Sarcoidosis*

c. *Treatment*

i- *Cytotoxic and immunosuppressive drugs*

e.g. steroids, azathioprine, vincristine and other drugs used in the treatment of cancer and for recipients of transplants.

These drugs frequently cause neutropenia and severe immunodeficiency

ii. *Radiotherapy*

(e.g. whole-body irradiation for patients receiving bone marrow transplants is an extreme example)

Instrumentation and surgery

These manipulations impair local mechanical barriers to infection and facilitate the invasion of organisms into the body, e.g. intravenous infusions and CVP lines, indwelling urinary catheters, tracheostomies and IPPR with use of ventilators and humidifiers.

Endogenous infection is most frequent with these invasive procedures but cross-infection is also common. The causative organisms often include multiple antibiotic-resistant strains of bacteria

Administration of broad-spectrum antibiotics

The giving of broad-spectrum antibiotics to patients with impaired host defences and/or recent instrumentation predisposes to superinfection by antibiotic-resistant strains of opportunist bacteria, such as *Kiebsiella, Pseudomonas* or Serraria, of fungi, such as *Candida.*

NB Immunosuppressed patient + ampicillin = *Klebsidla* infection (in many patients)

Structural damage to an organ or system

For example, a kidney damaged by calculi or a lung damaged by previous infection provides a nidus for bacteria and other organisms.

Foreign bodies

Implantation of foreign materials also provides a nidus for infection, often by organisms of low pathogenicity. For example, *Staph. epidermidis* and candida infections of arteriovenous shunts in renal dialysis patients, aortic dacron grafts, intravenous catheters, prosthetic heart valves, etc.

DIAGNOSIS OF OPPORTUNISTIC INFECTIONS

Clinical features are often lacking in immunocompromised patients with infection. Fever is frequently the only obvious feature. However, fever may also be due to non-infective causes including malignancy or drug reactions.

In all febrile patients' blood cultures, preferably two or three sets, should be collected before the start of prompt ‘blind’ chemotherapy.

If possible, a urine sample should also be collected for culture before treatment starts.
A clear bacteriological diagnosis, achieved in only 20-40% of patients, is valuable for subsequent optimal therapy.
Knowledge of the patient's clinical stares, chest X-ray findings, the types of ‘opportunistic conditions’ present, and the local prevalence of ‘opportunist’ organisms may cause certain infections to be suspected. The specimens required for each main type of infection arc included in *Table7.1.*
Diagnosis of the exact site of infection is frequently difficult in severely neutropenic patients. Signs of infection may be lacking due to deficiency in ‘pus’ cells and impaired inflammatory response. Common sites of infection that need to be considered include the lungs, pharynx and aesophagus, and pen-anal region. in some patients, bacteria from the faecal flora enter the blood from the normal gastrointestinal tract. Although symptoms or signs at a localized site, such as the perianal region, may be minimal, appropriate swabs should be collected in addition to blood and urine cultures. Physical examination of the chest and chest X-ray may also show only minimal abnormalities when the patient presents with fever, and sputum may not be produced.
A few days after cultures have been collected, and ‘blind’ antibiotic treatment started, there may be little evidence of any clinical response in some patients who have diffuse shadows apparent on a chest X-ray. Unusual opportunist organisms *(Tables 7.1—7.3)* need serious consideration in these circumstances.
Pneumocystic pneumonia is the most frequent opportunistic infection in patients with AIDS and the diagnosis of this condition in these patients is discussed further in Chapter 22. Diagnosis of *Pneumocystis* and other unusual opportunistic lung infections by open lung biopsy is probably the method of choice since adequate samples of tissue can then be examined by silver stains (such as Grocott’s and other stains) to see the characteristic pneumocystis cysts or fungi. Histology may also reveal infection by *Mycobacrerium tuberculosis* or cytomegalovirus. However, open lung biopsy is not practical in many patients and less invasive procedures such as fibre-optic bronchoscopy with the collection of bronchial washings or biopsy material may be considered. ‘Induced sputum’ samples can be examined by microscopy and culture for fungi and acid-fast bacilli. An immunofluorescent anti-pneumocystis stain or a Grocott’s silver stain of this sputum may reveal pneumocvstis infection.
Serological investigations for evidence of infection by *Condida albicans* (candida antigen), Aspergillus *furnigatus* (aspergillus antigen and precipitins), *Crvptococcus neoforinans* (cryptococcal antigen), cytomegalovirus (CMV,

CFT), *Legionella pneumophila* (fluorescent legionella antibodies), and other opportunist organisms are useful when paired sera show a rising ‘specific’ antibody titre of fourfold or greater, or a single high titre or a positive antigen test. A positive specific 1gM antibody result is particularly helpful if cytomegalovirus or legionella infection is suspected. However, these antibody tests are frequently unhelpful since ‘false negative’ or only low serum antibody titres result in some patients with established infections, because the immune response is too poor to generate significant antibody titres.
Molecular biology tests are likely to have an important role in future for the diagnosis of certain opportunistic infections such as pneumocystis and cytomegalovirus disease

TREATMENT OF OPPORTUNISTIC INFECTIONS

Initial treatment

The majority of infected compromised patients who acquire infections in hospital have bacterial infections which require prompt ‘blind’ treatment as soon as the cultures have been collected. Bactericidal antibiotic combinations using two drugs generally give the best results as the causative bacterial strains are likely to be sensitive to at least one of the antibiotics in the combination and, if sensitive to both the antibiotics, a synergic antibacterial effect may sometimes be achieved. The particular selection of ‘blind’ drugs depends on knowledge of the local prevalence of pathogens and their antibiotic sensitivity characteristics. *Pseudomonas aeruginosa* is an example of one organism which may frequently cause serious infections in neutropenic patients in one hospital centre, but not in another. (Considerations of recent positive cultures, such as a urine yielding growth of a *Kiebsiella* strain resistant to a cephalosporin, and of any recent antibiotics given should also be included when selecting ‘blind’ antibiotics for treating a febrile illness).
Selection of antibacterial agents from the following three groups has been recommended (to be given as combinations) for ‘blind therapy in the initial treatment of infections in netitropenic patients with leukaemia :

1. Aminoglycosides, such as genranhicin, netilmicin or amikacin - active against Gram-negative bacilli (and staphylococci).
2. Cephalosporins, such as cefuroxime, cefotaxime, ceftriaxone, or ceftazidime - active against Gram-negative bacilli and Gram-positive cocci *(see* p. 78).
3. ‘Anti-pseudomonas’ penicillins, such as ureidopenicillins, piperacillin or azlocillin - active against some *Pseudonumas* strains and other Gram-negative

bacilli (plus some anaerobes and streptococci) — or piperacillin plus clavulanic acid.

4. Fluoroquinolones such as ciprofloxacin may now also be considered, preferably in combination with another anti-pseudomonas antibiotic

Suitable combinations include an aminoglycoside plus an 'anti-pseudomonas' penicillin, or an aminoglycoside plus a cephalosporin. A cephalosporin which is resistant to most Gram-negative 13-lactamases and is active against *Pseudomonas aeruginosa,* such as ceftazidime, may also be suitable for treating some patients.

The problem of a patient still not responding to the antibiotic treatment has already been discussed. As mentioned, in a susceptible patient with possible lung infection the diagnosis of unusual opportunist organisms should be considered. Alternative explanations for an apparent non-response include the possibility of (I) a cryptic collection of 'pus' which might require drainage, (2) a contaminated intravascular line, especially with persisting Gram-positive infections, which requires removal, (3) superinfection by antibiotic-resistant organisms, (4) administration of inadequate doses of drugs or (5) profound granulocytopenia.

Granulocyte transfusions or/and colony-stimulating factor may be useful in some patients with persistent severe neutropenia (less than 500 granulocytes per mm^3) who develop septicaemia responding poorly to antibiotic therapy.

Anti-tuberculous drugs should be especially considered empirically in non-responding PUO cases who have been persistently immunosuppressed, including recipients of renal transplants, especially if fever presents longer than a week.

Antifungal chemotherapy

Fungal infections of the lungs (mainly *Aspergillus* or *Cryptococcus),* central nervous system, heart or other sites most frequently have an insidious onset in patients with lymphomas or prolonged immunosuppression and treatment is often only considered when the disease is well advanced with fatal consequences. Early recognition of the possibility of fungal infection and 'blind' empirical antifungal treatment is usually necessary without confirming the diagnosis microbiologically.

Amphotericin B is still the best agent to use for treating aspergillus, cryptococcal and candida systemic infections and is also given in 'blind' antifungal chemotherapy. An intravenous infusion in 5% dextrose is necessary, usually starting with a daily dose in an adult of 1.0 mg and increasing the dose progressively to 0.6 mg/kg per day during the next fortnight. Some authorities

recommend simultaneous mannitol administration to reduce the chances of nephrotoxicity developing. Febrile and other unwanted effects are common, but usually it is necessary to continue the treatment unless serious toxic effects develop. Combined treatment with amphotericin B and 5-fluorocytosine has been recommended for treating some serious yeast infections, e.g. *Candida albicans* oesophageal and bronchial infections with candidaemia, in an immunosuppressed patient. Specialist advice from experts with extensive experience in treating serious fungal infections should be sought at an early stage. Amphotericin B is recommended for treating disseminated cryptococcal infection in patients with AIDS.

Antiviral chemotherapy

Acute pneumonitis or disseminated lesions caused by varicella-zoster or herpes simplex viruses in immunosuppressed patients or patients with lvinphoma are rapidly fatal and require urgent systemic antiviral chemotherapy. Acycloguanosine (aciclovir) has produced dramatic clinical improvement in some patients. Herpes simplex encephalitis is often difficult to treat effectively *(see* Chapter 11).

Cytomegalovirus is a frequent cause of infection in immunosuppressed patients, but is usually associated with infection of only mild to moderate severity. Recently. ganciclovir or foscamet have proved useful for treating some serious cytomegalovirus infections.

Antipneumocystis chemotherapy

Prompt 'blind' antipneumocystis chemotherapy is occasionally indicated when interstitial pneumonitis is clinically and/or radiologically suspected. This is most likely to arise in patients with AIDS and in children with acute lymphoblastic leukaemia, lymphoina patients and recipients of transplants receiving prolonged immunosuppression. Pentamidine is often effective but is a toxic drug. Cotrimoxazole, given in high dosage, has now replaced pentamidine and is effective provided treatment is started in the early stages of the disease. Hypersensitivity reactions to cocrirnoxazole are more frequent in patients with AIDS than in other patients and the treatment of pneumocvstis pneumonia is discussed further in Chapter 22.

Antistrongyloides chemotherapy

Thiabendazole has been used successfully to treat pulmonary strongyloidiasis in immune-suppressed patients but may not be effective unless treatment is started early in the infection.

PREVENTION OF OPPORTUNISTIC INFECTIONS

Any impairment of general host defences by treatment is kept under regular review so that the lowest dosage of immunosuppressive drugs, such as steroids, are used for the shortest possible duration that is compatible with effective treatment of the non-infective condition. The main preventive measures include the following.

1. High standards of asepsis and antisepsis

Special care is necessary to avoid iniection when putting up intravenous infusions, inserting CVP lines, performing peritoneal dialysis or other forms of instrumentation.

2. Agreed antimicrobial drug policy and prophylaxis

a. Systemic drugs

The use of systemic broad-spectrum antibiotics in special units should be reduced to a minimum. However, some authorities have suggested the use of ciprofloxacin for the prophylaxis of bacterial infections in neutropenic patients. This antibiotic is not active against *Bacteroides* in the normal flora of the gut so 'colonization resistance' against some hospital pathogens may be maintained. Constant bacteriological monitoring of the pathogcns isolated from clinical specimens from patients in special units is necessary with particular attention to the current antibiotic sensitivity patterns. It may be necessary to temporarilv 'ban' the use of certain antibiotics in a special unit which are associated with a high incidence of antibiotic resistance in the unit. With boric marrow transplant patients, prolonged prophvlaxis with cotrimoxazole may be indicated to prevent pneumocystis lung infection.

b. Oral non_absorbable drugs

Adminisrratibn or oral non-absorbable drugs may help to prevent septicaemia and anorectal abscesses in patients during the treatment of acute leukaemia while severe neutropenia is occurring. Various mixtures have been recommended including FRACON (a combination of framycetin , colistin and nystatin, used in Britain). These oral drugs (oasionally supplemented by sterile topical antiseptics, such as chlorhexidine, applied to the external surfaces including the perineum and lower genital tract in the female) are given to reduce the gut flora which is the usual source of 'self-infections' in patients with granulocyte counts less than 500 per mm.

c. Prophylactic anti—tuberculous or antihelminth, drugs

In persistently immunosuppressed patients who are known to have had TB in the past, or who have come from a strongyloides endemic geographical area,

there is a reasonable indication for prophylaxis with anti-tuberculous drugs or thiahendazole, respectively.

3. Protective isolation

A single room is preferable for leukaemic or transplant patients expected to develop severe neutropenia during treatment and protective isolation procedures are desirable.

The hospital staff atending the compromised patient should not also nurse other patients with TB or varicella-zoster infections, unless they are definitely known to have immunity to these infections. These infected patients should be adequately separated from compromised patients. If an accidental contact occurs between the susceptible compromised patient and a person with measles or varicella-zoster infection, prophylaxis with the appropriate immunoglobulin should immediately be given. In the USA, varicella-zoster susceptible staff may now be given varicellazoster vaccine before caring for immunocompromised patients.

Patients who have severe and persistent immunodeficiency states or who are being immunosuppressed, including irradiated and immunosuppressed recipients of bone marrow transplants, may require complete positive pressure protective isolation. Patients with malignant disease who are having anticancer chemotherapy should not be given salads or uncooked vegetables which may be a source of Gram-negative bacilli that may colonize the gut.

Sterilization (Microbiology)

Sterilization (or **sterilisation**) refers to any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses, prions and spore forms etc.) from a surface, equipment, foods, medications, or biological culture medium. Sterilization can be achieved through application of heat, chemicals, irradiation, high pressure or filtration.

Medicine and surgery

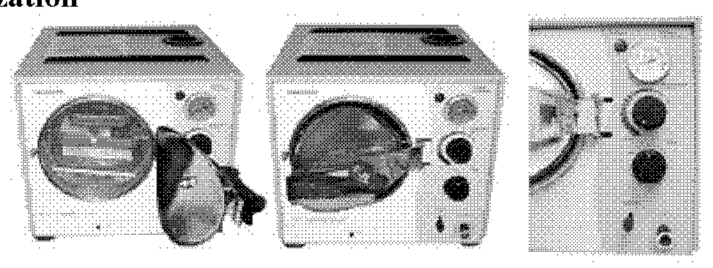
In general, surgical instruments and medications that enter an already sterile part of the body (such as the blood, or beneath the skin) must have a high sterility assurance level. Examples of such instruments include scalpels, hypodermic needles and artificial pacemakers. This is also essential in the manufacture of parenteral pharmaceuticals.

Heat sterilization of medical instruments is known to have been used in Ancient Rome, but it mostly disappeared throughout the Middle Ages resulting in significant increases in disability and death following surgical procedures.

Preparation of injectable medications and intravenous solutions for fluid replacement therapy requires not only a high sterility assurance level, but well-designed containers to prevent entry of adventitious agents after initial sterilization.

Heat sterilization

Steam sterilization



Front-loading autoclaves are very common.

A widely-used method for heat sterilization is the autoclave. Autoclaves commonly use steam heated to 121 °C or 134 °C. To achieve sterility a holding

time of atleast 15 minutes at 121 °C or 3 minutes at 134 °C is required. As items such as, liquids and instruments packed in layers of cloth may take longer to reach the required temperature than the steam solid instruments additional sterilising time is usually required. After sterilization, autoclaved liquids must be cooled slowly to avoid boiling over when the pressure is released.

Proper autoclave treatment will inactivate all fungi, bacteria, viruses and also bacterial spores, which can be quite resistant. It will not necessarily eliminate all prions.

For prion elimination, various recommendations state 121–132 °C (270 °F) for 60 minutes or 134 °C (273 °F) for at least 18 minutes. The prion that causes the disease scrapie (strain 263K) is inactivated relatively quickly by such sterilization procedures; however, other strains of scrapie, as well as strains of CJD and BSE are more resistant. Using mice as test animals, one experiment showed that heating BSE positive brain tissue at 134-138 °C (273-280 °F) for 18 minutes resulted in only a 2.5 log decrease in prion infectivity. (The initial BSE concentration in the tissue was relatively low). For a significant margin of safety, cleaning should reduce infectivity by 4 logs, and the sterilization method should reduce it a further 5 logs.

To ensure the autoclaving process was able to cause sterilization, most autoclaves have meters and charts that record or display pertinent information such as temperature and pressure as a function of time. Indicator tape is often placed on packages of products prior to autoclaving. A chemical in the tape will change color when the appropriate conditions have been met. Some types of packaging have built-in indicators on them.

Biological indicators ("bioindicators") can also be used to independently confirm autoclave performance. Simple bioindicator devices are commercially available based on microbial spores. Most contain spores of the heat resistant microbe *Bacillus stearothermophilus*, among the toughest organisms for an autoclave to destroy. Typically these devices have a self-contained liquid growth medium and a growth indicator. After autoclaving an internal glass ampule is shattered, releasing the spores into the growth medium. The vial is then incubated (typically at 56 °C (132 °F)) for 48 hours. If the autoclave destroyed the spores, the medium will remain its original color. If autoclaving was unsuccessful the *B. sterothermophilus* will metabolize during incubation, causing a color change during the incubation.

For effective sterilization, steam needs to penetrate the autoclave load uniformly, so an autoclave must not be overcrowded, and the lids of bottles and containers must be left ajar. During the initial heating of the chamber, residual

air must be removed. Indicators should be placed in the most difficult places for the steam to reach to ensure that steam actually penetrates there.

For autoclaving, as for all disinfection of sterilization methods, cleaning is critical. Extraneous biological matter or grime may shield organisms from the property intended to kill them, whether it physical or chemical. Cleaning can also remove a large number of organisms. Proper cleaning can be achieved by physical scrubbing. This should be done with detergent and warm water to get the best results. Cleaning instruments or utensils with organic matter, cool water must be used because warm or hot water may cause organic debris to coagulate. Treatment with ultrasound or pulsed air can also be used to remove debris.

Other methods

Other heat methods include flaming, incineration, boiling, tindalization, and using dry heat.

Flaming is done to loops and straight-wires in microbiology labs. Leaving the loop in the flame of a Bunsen burner or alcohol lamp until it glows red ensures that any infectious agent gets inactivated. This is commonly used for small metal or glass objects, but not for large objects (see Incineration below). However, during the initial heating infectious material may be "sprayed" from the wire surface before it is killed, contaminating nearby surfaces and objects. Therefore, special heaters have been developed that surround the inoculating loop with a heated cage, ensuring that such sprayed material does not further contaminate the area.

Incineration will also burn any b organism to ash. It is used to sanitize medical and other biohazardous waste before it is discarded with non-hazardous waste.

Boiling in water for 15 minutes will kill most vegetative bacteria and viruses, but boiling is ineffective against prions and many bacterial and fungal spores; therefore boiling is unsuitable for sterilization. However, since boiling does kill most vegetative microbes and viruses, it is useful for reducing viable levels if no better method is available. Boiling is a simple process, and is an option available to most anyone most anywhere, requiring only water, enough heat, and a container that can withstand the heat; however, boiling can be hazardous and cumbersome.

Tindalization /Tyndallization named after John Tyndall is a lengthy process designed to reduce the level of activity of sporulating bacteria that are left by a simple boiling water method. The process involves boiling for a period (typically 20 minutes) at atmospheric pressure, cooling, incubating for a day, boiling, cooling, incubating for a day, boiling, cooling, incubating for a day, and finally boiling again. The three incubation periods are to allow heat-

resistant spores surviving the previous boiling period to germinate to form the heat-sensitive vegetative (growing) stage, which can be killed by the next boiling step. This is effective because many spores are stimulated to grow by the heat shock. The procedure only works for media that can support bacterial growth - it will not sterilize plain water. Tindalization/tyndallization is ineffective against prions.

Dry heat can be used to sterilize items, but as the heat takes much longer to be transferred to the organism, both the time and the temperature must usually be increased, unless forced ventilation of the hot air is used. The standard setting for a hot air oven is at least two hours at 160 °C (320 °F). A rapid method heats air to 190 °C (374 °F) for 6 minutes for unwrapped objects and 12 minutes for wrapped objects. Dry heat has the advantage that it can be used on powders and other heat-stable items that are adversely affected by steam (for instance, it does not cause rusting of steel objects).

Prions can be inactivated by immersion in sodium hydroxide (NaOH 0.09N) for two hours plus one hour autoclaving (121 °C/250 °F). Several investigators have shown complete (>7.4 logs) inactivation with this combined treatment. However, sodium hydroxide may corrode surgical instruments, especially at the elevated temperatures of the autoclave.

Chemical sterilization

Chemicals are also used for sterilization. Although heating provides the most reliable way to rid objects of all transmissible agents, it is not always appropriate, because it will damage heat-sensitive materials such as biological materials, fiber optics, electronics, and many plastics.

Ethylene oxide (EO or EtO) gas is commonly used to sterilize objects sensitive to temperatures greater than 60 °C such as plastics, optics and electrics.

Ethylene oxide treatment is generally carried out between 30 °C and 60 °C with relative humidity above 30% and a gas concentration between 200 and 800 mg/L for at least three hours. Ethylene oxide penetrates well, moving through paper, cloth, and some plastic films and is highly effective. Ethylene oxide is the most common sterilization method, used for over 70% of total sterilizations, and for 50% of all disposable medical devices.

Ethylene oxide sterilizers are used to process sensitive instruments which cannot be adequately sterilized by other methods. EtO can kill all known viruses, bacteria and fungi, including bacterial spores and is satisfactory for most medical materials, even with repeated use.

However it is highly flammable, and requires a longer time to sterilize than any heat treatment. The process also requires a period of post-sterilization

aeration to remove toxic residues. This method has drawbacks inherent to the use of large amounts of sterilant being released into a large space, including air contamination produced by CFCs and/or large amounts of EtO residuals, flammability and storage issues calling for special handling and storage, operator exposure risk and training costs.

The two most important ethylene oxide sterilization methods are:

(1) Gas chamber method. To benefit from economies of scale, EtO has traditionally been delivered by flooding a large chamber with a combination of EtO and other gases used as dilutants (usually CFCs or carbon dioxide).

(2) Micro-dose method. a micro-dose sterilization method was developed in the late 1950s, using a specially designed bag to eliminate the need to flood a larger chamber with EtO. This method is also known as gas diffusion sterilization, or bag sterilization. This method minimize the use of gas.

Bacillus subtilis, a very resistant organism, is used as a rapid biological indicator for EO sterilizers. If sterilization fails, incubation at 37 °C causes a fluorescent change within four hours, which is read by an auto-reader. After 96 hours, a visible color change occurs. Fluorescence is emitted if a particular (EO resistant) enzyme is present, which means that spores are still active. The color change indicates a pH shift due to bacterial metabolism. The rapid results mean that the objects treated can be quarantined until the test results are available.

Ozone is used in industrial settings to sterilize water and air, as well as a disinfectant for surfaces. It has the benefit of being able to oxidize most organic matter. On the other hand, it is a toxic and unstable gas that must be produced on-site, so it is not practical to use in many settings.

Chlorine bleach is another accepted liquid sterilizing agent. Household bleach consists of 5.25% sodium hypochlorite. It is usually diluted to 1/10 immediately before use; however to kill *Mycobacterium tuberculosis* it should be diluted only 1/5. The dilution factor must take into account the volume of any liquid waste that it is being used to sterilize.[4] Bleach will kill many organisms immediately, but for full sterilization it should be allowed to react for 20 minutes. Bleach will kill many, but not all spores. It is highly corrosive and may corrode even stainless steel surgical instruments.

Glutaraldehyde and formaldehyde solutions (also used as fixatives) are accepted liquid sterilizing agents, provided that the immersion time is sufficiently long. To kill all spores in a clear liquid can take up to 12 hours with glutaraldehyde and even longer with formaldehyde. The presence of solid particles may lengthen the required period or render the treatment ineffective. Sterilization of blocks of tissue can take much longer, due to the time required

for the fixative to penetrate. Glutaraldehyde and formaldehyde are volatile, and toxic by both skin contact and inhalation. Glutaraldehyde has a short shelf life (<2 weeks), and is expensive. Formaldehyde is less expensive and has a much longer shelf life if some methanol is added to inhibit polymerization to paraformaldehyde, but is much more volatile. Formaldehyde is also used as a gaseous sterilizing agent; in this case, it is prepared on-site by depolymerization of solid paraformaldehyde. Many vaccines, such as the original Salk polio vaccine, are sterilized with formaldehyde.

Ortho-phthalaldehyde (OPA) is a chemical sterilizing agent that received Food and Drug Administration (FDA) clearance in late 1999. Typically used in a 0.55% solution, OPA shows better myco-bactericidal activity than glutaraldehyde. It also is effective against glutaraldehyde-resistant spores. OPA has superior stability, is less volatile, and does not irritate skin or eyes, and it acts more quickly than glutaraldehyde. On the other hand, it is more expensive, and will stain proteins (including skin) gray in color.

Hydrogen peroxide is another chemical sterilizing agent. It is relatively non-toxic once diluted to low concentrations (although a dangerous oxidizer at high concentrations), and leaves no residue.

Low Temperature Plasma sterilization chambers use hydrogen peroxide vapor to sterilize heat-sensitive equipment such as rigid endoscopes. A recent model can sterilize most hospital loads in as little as 20 minutes. The Sterrad has limitations with processing certain materials such as paper/linens and long thin lumens. Paper products cannot be sterilized in the Sterrad system because of a process called cellulostics, in which the hydrogen peroxide would be completely absorbed by the paper product.

Hydrogen peroxide and formic acid are mixed as needed in the Endoclens device for sterilization of endoscopes. This device has two independent asynchronous bays, and cleans (in warm detergent with pulsed air), sterilizes and dries endoscopes automatically in 30 minutes. Studies with synthetic soil with bacterial spores showed the effectiveness of this device.

Dry sterilization process (DSP) uses hydrogen peroxide at a concentration of 30-35% under low pressure conditions. This process achieves bacterial reduction of 10^{-6}...10^{-8}. The complete process cycle time is just 6 seconds, and the surface temperature is increased only 10-15 °C (18 to 27 °F). Originally designed for the sterilization of plastic bottles in the beverage industry, because of the high germ reduction and the slight temperature increase the dry sterilization process is also useful for medical and pharmaceutical applications.

Peracetic acid (0.2%) is used to sterilize instruments in the Steris system.

Prions are highly resistant to chemical sterilization. Treatment with aldehydes (e.g., formaldehyde) have actually been shown to increase prion resistance. Hydrogen peroxide (3%) for one hour was shown to be ineffective, providing less than 3 logs (10^{-3}) reduction in contamination. Iodine, formaldehyde, glutaraldehyde and peracetic acid also fail this test (one hour treatment). Only chlorine, a phenolic compound, guanidinium thiocyanate, and sodium hydroxide (NaOH) reduce prion levels by more than 4 logs. Chlorine and NaOH are the most consistent agents for prions. Chlorine is too corrosive to use on certain objects. Sodium hydroxide has had many studies showing its effectiveness.

Radiation sterilization

Methods exist to sterilize using radiation such as X-rays, gamma rays, or subatomic particles.

- Gamma rays are very penetrating and are commonly used for sterilization of disposable medical equipment, such as syringes, needles, cannulas and IV sets. Gamma radiation requires bulky shielding for the safety of the operators; they also require storage of a radioisotope (usually Cobalt-60), which continuously emits gamma rays (it cannot be turned off, and therefore always presents a hazard in the area of the facility).
- X-rays are less penetrating than gamma rays and tend to require longer exposure times, but require less shielding, and are generated by an X-ray machine that can be turned off for servicing and when not in use.
- Ultraviolet light irradiation (UV, from a germicidal lamp) is useful only for sterilization of surfaces and some transparent objects. Many objects that are transparent to visible light absorb UV. UV irradiation is routinely used to sterilize the interiors of biological safety cabinets between uses, but is ineffective in shaded areas, including areas under dirt (which may become polymerized after prolonged irradiation, so that it is very difficult to remove). It also damages many plastics, such as polystyrene foam.

Ultraviolet Germicidal Irradiation

- Subatomic particles may be more or less penetrating, and may be generated by a radioisotope or a device, depending upon the type of particle.

Irradiation with X-rays or gamma rays does not make materials radioactive. Irradiation with particles may make materials radioactive, depending upon the type of particles and their energy, and the type of target material: neutrons and very high-energy particles can make materials radioactive, but have good penetration, whereas lower energy particles (other than neutrons) cannot make materials radioactive, but have poorer penetration.

Irradiation is used by the United States Postal Service to sterilize mail in the Washington, DC area. Some foods (e.g. spices, ground meats) are irradiated for sterilization (see food irradiation).

Sterile filtration

Clear liquids that would be damaged by heat, irradiation or chemical sterilization can be sterilized by mechanical filtration. This method is commonly used for sensitive pharmaceuticals and protein solutions in biological research. A filter with pore size 0.2 µm will effectively remove bacteria. If viruses must also be removed, a much smaller pore size around 20 nm is needed. Solutions filter slowly through membranes with smaller pore diameters. Prions are not removed by filtration. The filtration equipment and the filters themselves may be purchased as presterilized disposable units in sealed packaging, or must be sterilized by the user, generally by autoclaving at a temperature that does not damage the fragile filter membranes. To ensure sterility, the filtration system must be tested to ensure that the membranes have not been punctured prior to or during use.

To ensure the best results, pharmaceutical sterile filtration is performed in a room with highly filtered air (HEPA filtration) or in a laminar flow cabinet or "flowbox", a device which produces a laminar stream of HEPA filtered air.

Antiseptic solution uses

Phonelics:-

Examples Compounds :-

- clearsol
- Hycolin

Active against:-

- Gram negative bacteria
- Gram positive bacteria
- Mycobacteria

Little activity :

- endospores.

viruses e.g. hepatitis B virus.

Effective concentration 2 %

Perochlorites and chlorines

- chloros
- Domestos
- Diversol Bx
- Sterite
- Milton

Active against:-

- Bacteria
- Viruses

Less active against:-

Mycobacteria

For use in virology and for spilled blood:-

The effective concentration 10.000 parts per million for AID, HBs Ag 1000 P.P for cleaning.

Glutaraldehyde and formaldehyde :-

- Asep.
- Cidex
- Clinicide
- 3 M
- Totacide
- Triocide

Working concentration 2 %

- It is bactericidal and virucidal but less effective against mycobacteria.
- Effective concentration 2 %

Ethyl alcohol:-

Ethanol 70 %

Active against; bacteria and Acid – fast bacilli.

Inactive against spores

Quaternary ammonium compounds

active against viruses, spores , mycobacteria and a non enveloped viruses.

Guanides:-

-chlorhexidine

activity against staph .aureus, moderate Gram – negative bacilli, less active against Pseudomonas which can grow in chlorhexidine solution.

Iodophors and Iodine:

Active against vegetative bacteria, some sporicidal, some antiviral and antifungal.

VIRAL HEPATITIS

The term VIRAL HEPATITIS is usually used to describe infections caused by agents whose primary tissue tropism is the liver.
To date, at least five hepatitis viruses have been recognized, and these have been named:-
Hepatitis A, B, C, D and E.
Acute hepatitis may also occur as part of the clinical course of a number of viral infections, including human cytomegalovirus, Epstein-Barr virus, herpes simplex virus, yellow fever virus and rubella.
Viral hepatitis can be classified according to mode of transmission
ENTERICALLY TRANSMITTED HEPATITIS: A and E
PARENTERALLY TRANSMITTED HEPATITIS B, C, D and G
HEPATITIS A VIRUS

Virology

RNA Picornavirus

- Single serotype worldwide
- Acute disease and asymptomatic infection
- No chronic infection
- Protective antibodies develop in response to infection - confers lifelong immunity

Hepatitis A is caused by HAV, a 27-nm ribonucleic acid (RNA) agent that is classified as a picornavirus. Only one serotype has been observed among HAV isolates collected from various parts of the world. HAV causes both acute disease and asymptomatic infection. HAV does not cause chronic infection. Total antibody to HAV develops in response to infection and confers lifelong immunity from future HAV infection.

Mode of transmission:

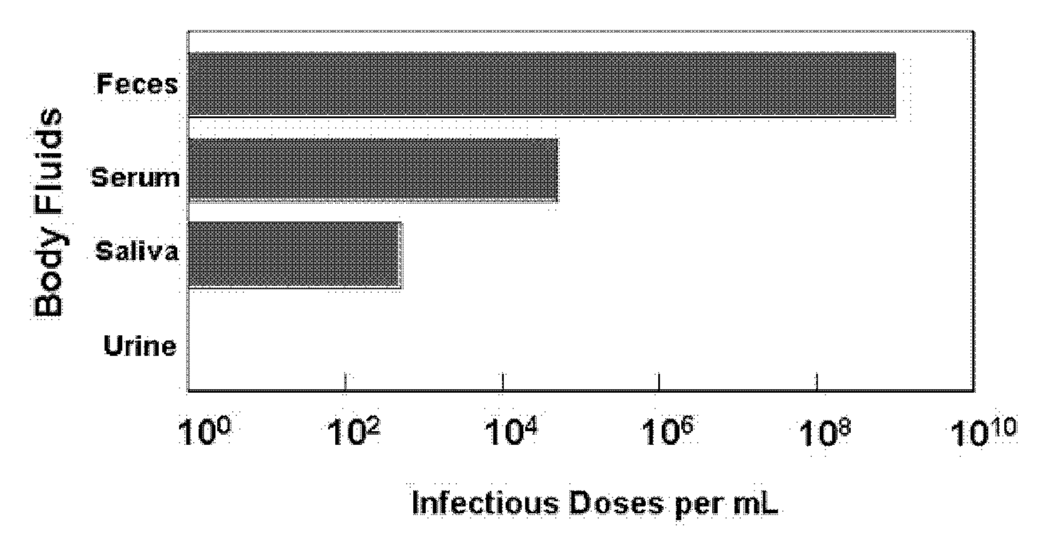
Feces can contain up to 108 infectious virions per milliliter and are the primary source of HAV. Viremia occurs during the preclinical and clinical phases of illness, and HAV has been transmitted by transfusion (before screening of blood and blood products for HAV was initiated) and by injection drug use. Virus has also been found in saliva and urine during the incubation period in experimentally infected animals, but transmission by saliva or urine has not been reported to occur.

The commenst routes of transmission

- Close personal contact (e.g., household contact, sex contact, child day-care centers)

- Contaminated food, water (e.g., infected food handlers)
- Blood exposure (rare) (e.g., injection drug use, rarely by transfusion)

Transmission of HAV generally occurs when susceptible persons put anything in their mouths that has been contaminated with the feces of an infected person. Close personal contact is the most common mode of HAV transmission, as demonstrated by infections among household and sex contacts of persons with hepatitis A and among children in day-care center outbreaks. Contaminated food and water can also serve as vehicles of HAV transmission. HAV transmission can occur when an infected food handler directly handles uncooked or cooked foods. Outbreaks have also been reported in association with foods contaminated before wholesale distribution, such as fresh vegetables contaminated at the time of harvesting or processing. HAV transmission can occur as a result of blood exposures such as injecting drug use or blood transfusion because viremia can occur prior to the onset of illness in infected persons. Screening of blood products for HAV has essentially eliminated the already extremely low risk associated with transfusion.



Source: Viral Hepatitis and Liver Disease 1984;9-22
J Infect Dis 1989;160:887-890

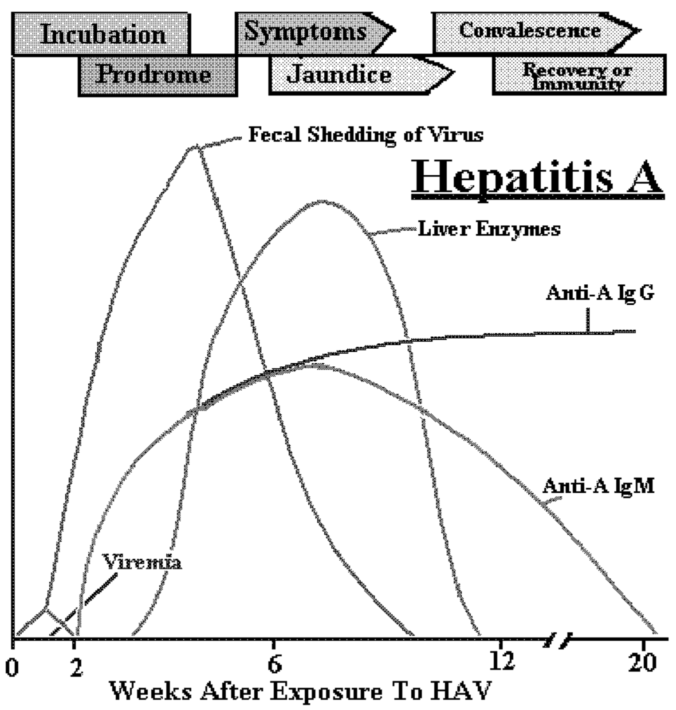
Incubation Period:

The average incubation period for hepatitis A is 30 days, with a range of 15 to 50 days.

Clinical Picture:

Patients characteristically have abrupt onset of symptoms which can include fever, malaise, anorexia, nausea, abdominal discomfort, dark urine, and jaundice. The severity of clinical disease associated with HAV infection increases with increasing age; jaundice occurs among less than 10% of children younger than 6 years of age, 40%-50% of older children, and 70%-80% of adults.

Complications of hepatitis A include fulminant hepatitis, in which the case fatality rate can be greater than 50% despite medical interventions such as liver transplantation; cholestatic hepatitis, with very high bilirubin levels that can persist for months; and relapsing hepatitis, in which exacerbations can occur weeks to months after apparent recovery. Chronic infection does not occur following HAV infection.



Laboratory Diagnosis Of Hepatitis A

-The diagnosis of acute HAV infection is confirmed during the acute or early convalescent phase of infection by the presence of IgM antibodies to HAV (IgM anti-HAV). IgM anti-HAV is generally present 5-10 days before the onset

of symptoms and is no longer detectable in the vast majority of patients 6 months later.
- anti-HAV IgG, which also appears early in the course of infection, remains detectable for the lifetime of the individual and confers lifelong protection against infection. Commercial tests are available for the detection of IgM and total (IgM and IgG) anti-HAV in serum.
-In infected persons, HAV replicates in the liver, is excreted in bile, and is shed in the stool. Peak infectivity occurs during the 2-week period before onset of jaundice or elevation of liver enzymes, when the concentration of virus in stool is highest. The concentration of virus in stool declines after jaundice appears. Children and infants can shed HAV for longer periods than adults, up to several months after the onset of clinical illness. Chronic shedding of HAV in feces does not occur; however, shedding can occur in persons who have relapsing illness. Viremia occurs soon after infection and persists through the period of liver enzyme (alanine aminotransferase [ALT]) elevation.
HAV RNA can be detected in the blood and stool of most persons during the acute phase of infection by using nucleic acid amplification methods, such as PCR, and nucleic acid sequencing has been used to determine the relatedness of HAV isolates. These methods, however, are available in only a limited number of research laboratories and are not used generally for diagnostic purposes.

-Elevated liver function tests begin from 4th weeks of infection and reach maximum elevation in 8th weeks after infection and return to normal within 12th weeks after infection notice that jaundice incidence differs according to age of infection-

• Jaundice by age group:	<6	yrs <10%
	6-14	yrs 40%-50%
	>14 yrs	70%-80%

PREVENTING HEPATITIS A
- Hygiene (e.g., hand washing)
- Sanitation (e.g., clean water sources)
- Hepatitis A vaccine (pre-exposure)
- Immune globulin (pre- and post-exposure)

Notes:
Good hygienic practices and adequate sanitation are important elements in the prevention of HAV infection, particularly in the developing world. However, hepatitis A vaccine is the key component in the overall strategy to prevent HAV

infection in the United States. Immune globulin is also available for pre-exposure and post-exposure prophylaxis.

PREPARATION OF INACTIVATED HEPATITIS A VACCINES

- Cell culture adapted virus grown in human fibroblasts
- Purified product inactivated with formalin
- Adsorbed to aluminum hydroxide adjuvant

Notes:

In the United States, highly immunogenic and efficacious inactivated hepatitis A vaccines were first licensed in 1995 by the Food and Drug Administration (FDA). These vaccines are prepared by methods similar to those used for inactivated poliovirus vaccine. Cell culture-adapted virus is propagated in human fibroblasts, purified from cell lysates by ultrafiltration and exclusion gel chromatography or other methods, inactivated with formalin, and adsorbed to an aluminum hydroxide adjuvant.

DURATION OF PROTECTION AFTER HEPATITIS A VACCINATION

- Persistence of antibody
  - At least 5-8 years among adults and children
- Efficacy
  - No cases in vaccinated children at 5-6 years of follow-up
- Mathematical models of antibody decline suggest protective antibody levels persist for at least 20 years
- Other mechanisms, such as cellular memory, may contribute

Notes:

Among adults and children, studies have demonstrated that detectable antibody persists for at least 5-8 years after completing the vaccination series. Although data regarding long-term efficacy are limited, no cases among vaccinated children were observed in one community at 5-6 years of follow-up. Estimates of antibody persistence derived from mathematical models of antibody decline indicate that protective levels of anti-HAV persist for at least 20 years. Whether other mechanisms such as cellular memory also contribute to long-term protection is unknown.

FACTORS ASSOCIATED WITH DECREASED IMMUNOGENICITY TO HEPATITIS A VACCINE

- Decreased antibody concentration:
  - Concurrent administration of IG
  - Presence of passively-transferred maternal antibody
  - Age

- Chronic liver disease
- Decreased seroconversion rate:
  - HIV infection
  - May be related to degree of immunosuppression
  - Liver transplantation

Notes:

The presence of anti-HAV at the time of vaccination appears to blunt the immune response. Administration of immune globulin (IG) concurrently with the first dose of hepatitis A vaccine did not decrease the proportion of adults who developed protective levels of antibody compared with adults who had been administered hepatitis A vaccine alone, but the geometric mean antibody concentrations (GMCs) among adults who received IG were lower 1 month after completion of the vaccination series than the GMCs of any adults who had been administered hepatitis A vaccine alone. The reduced immunogenicity of hepatitis A vaccine that occurs with concurrent administration of IG does not appear to be clinically significant. IG and hepatitis A vaccine can be given concurrently if indicated.

Reduced vaccine immunogenicity also has been observed in infants who had passively-transferred antibody because of prior maternal HAV infection and were administered hepatitis A vaccine according to a number of different schedules. In most studies, all infants developed protective levels of antibody, but the final GMCs were approximately 1/3 to 1/10 those of infants born to anti-HAV-negative mothers.

Based on limited data, final antibody concentrations might be lower among older vaccinated persons.

Vaccination of adults with chronic liver disease of viral or nonviral etiology produced seroprotection rates similar to those observed in healthy adults. Final antibody concentrations, however, were substantially lower for each group of patients with chronic liver disease than for healthy adults.

Hepatitis B

HBV(DNA) from **Hepadna virus**

Incubation Peroid:

6 weeks-6 months

Mode Of Transmission:

Parenteral, sexual, vertical

Clinical Course:

Prolonged and more severe than A

Laboratory Diagnosis:

-Elevated ALT,AST from 10-100 folds **Acute infection with resolution**

-Viralantigens:

1) **Surface antigen (HBsAg)** is secreted in excess into the blood as 22 nm spheres and tubules. Its presence in serum indicates that virus replication is occurring in the liver

2) **'e' antigen (HBeAg)** secreted protein is shed in small amounts into the blood. Its presence in serum indicates that a high level of viral replication is occurring in the liver

3) **core** antigen (HBcAg) core protein is not found in blood

-Antibody response:

1) **Surface antibody** (anti-HBs) becomes detectable late in convalescence, and indicates immunity following infection. It remains detectable for life and is not found in chronic carriers (see below).

2) **e antibody** (anti-HBe) becomes detectable as viral replication falls. It indicates low infectivity in a carrier.

3) **Core IgM** rises early in infection and indicates recent infection

4) **Core IgG** rises soon after IgM, and remains present for life in both chronic carriers as well as those who clear the infection. Its presence indicates exposure to HBV.

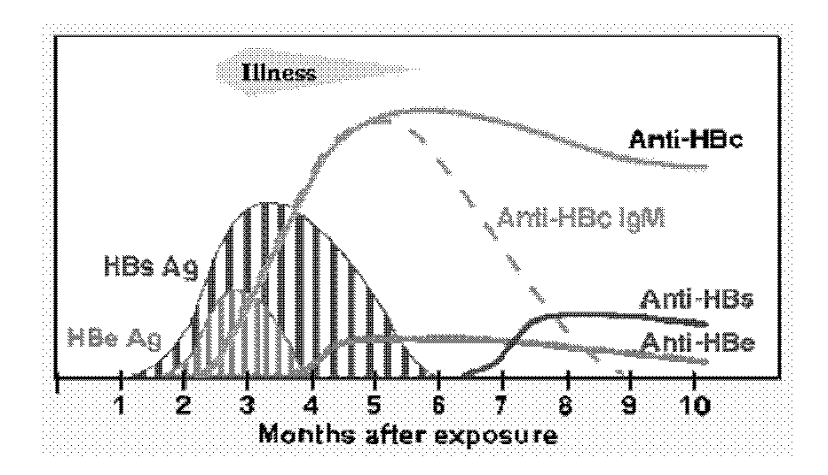


Figure: Acute Hepatitis B Infection

Chronic hepatitis B

Persistance of surface antigen and prolonged persistence of e antigen.

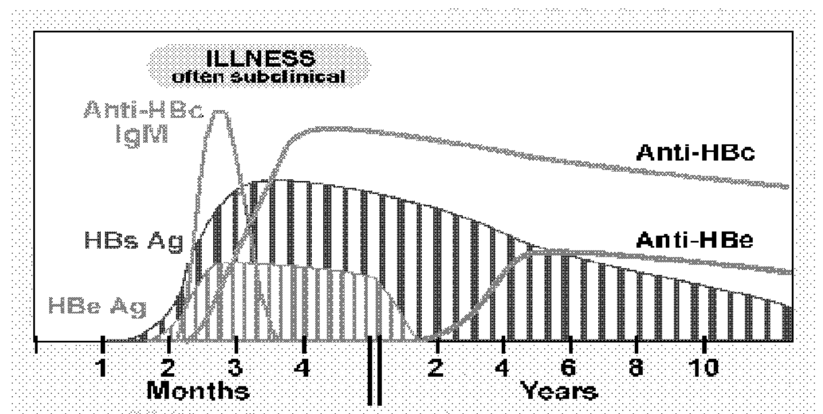


Figure: Chronic Hepatitis B Infection

Hepatitis C Virus:

HCV(RNA) from **Togavirus** related to the Flavi and Pesti viruses.

Features of Hepatitis C Virus Infection

Incubation period	Average 6-7 weeks Range 2-26 weeks
Acute illness (jaundice)	Mild (≤20%)
Case fatality rate	Low
Chronic infection*	60%-85%
Chronic hepatitis*	10%-70% (most asymptomatic)
Cirrhosis*	<5%-20%
Mortality from CLD	1%-5%

*Age related

Exposures Known to be Associated With HCV Infection

- Injecting drug use
- Transfusion, transplant from infected donor
- Occupational exposure to blood

- Mostly needle sticks
- Case reports of transmission from blood splash to eye; one from exposure to non-intact skin

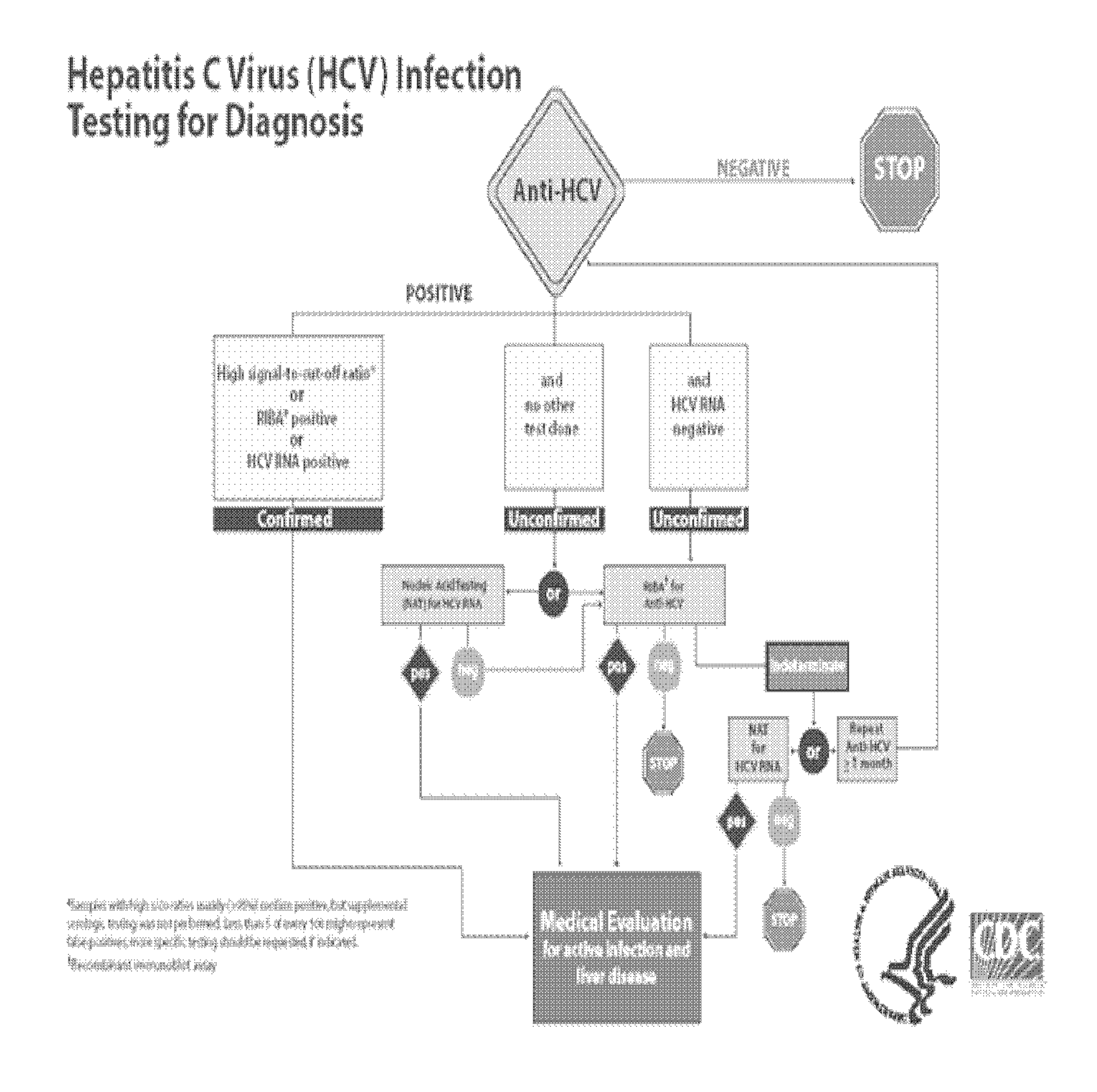
-Prevalence 1-2% among health care workers
Lower than adults in the general population
10 times lower than for HBV infection

- Iatrogenic (unsafe injections)
- Birth to HCV-infected mother

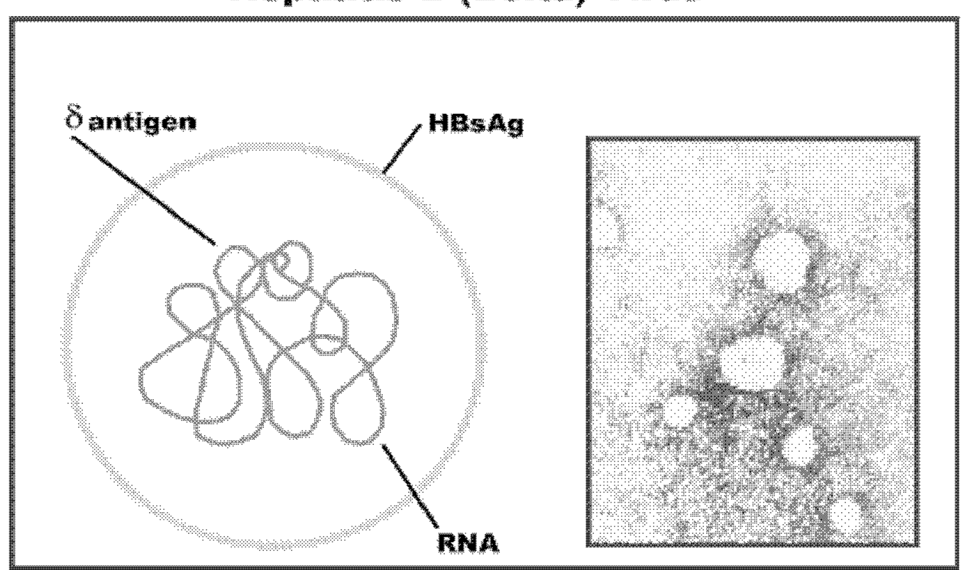
Average rate of infection 4%
Higher (19%) if woman co-infected with HIV
Role of viral titer unclear
No association with
Delivery method
Breastfeeding
Infected infants do well
Severe hepatitis is rare

- Sex with infected partner
- Household Transmission of HCV
- Rare but not absent
- Could occur through percutaneous/mucosal exposures to blood
    - Contaminated equipment used for home therapies
        - IV therapy, injections
    - Theoretically through sharing of contaminated personal articles (razors, toothbrushes)

Laboratory Diagnosis Of Hepatitis C:
-Mild elevation of ALT,AST
-With fluctuation in AST(surrogate Marker of chronic hepatitis C).
1)Serology
1-HCV-specific IgG indicates exposure, not infectivity
2) PCR detects viral genome in patient's serum
3) Quantitative PCR to detect viral load response to therapy


Hepatitis C Virus (HCV) Infection
Testing for Diagnosis
Anti-HCV
NEGATIVE
STOP
POSITIVE
High signal-to-cut-off ratio*
or
RIBA† positive
or
HCV RNA positive
Confirmed
and
no other
test done
Unconfirmed
and
HCV RNA
negative
Unconfirmed
or
pos
neg
pos
neg
STOP
NAT
for
HCV RNA
or
Repeat
Anti-HCV
pos
neg
STOP
Medical Evaluation
for active infection and
liver disease

Hepatitis D (Delta) Virus



HDV is a defective single-stranded RNA virus that requires the helper function of HBV to replicate. HDV requires HBV for synthesis of envelope protein composed of HBsAg, which is used to encapsulate the HDV genome.

Hepatitis D - Clinical Features

- Co infection
  - severe acute disease
  - low risk of chronic infection
- Super infection
  - usually develop chronic HDV infection high
  - risk of severe chronic liver disease

Notes:

HDV infection can be acquired either as a co-infection with HBV or as a super infection of persons with chronic HBV infection. Persons with HBV-HDV co-infection may have more severe acute disease and a higher risk of fulminant hepatitis (2%-20%) compared with those infected with HBV alone; however, chronic HBV infection appears to occur less frequently in persons with HBV-HDV co-infection. Chronic HBV carriers who acquire HDV super infection usually develop chronic HDV infection. In long-term studies of chronic HBV carriers with HDV superinfection, 70%-80% have developed evidence of

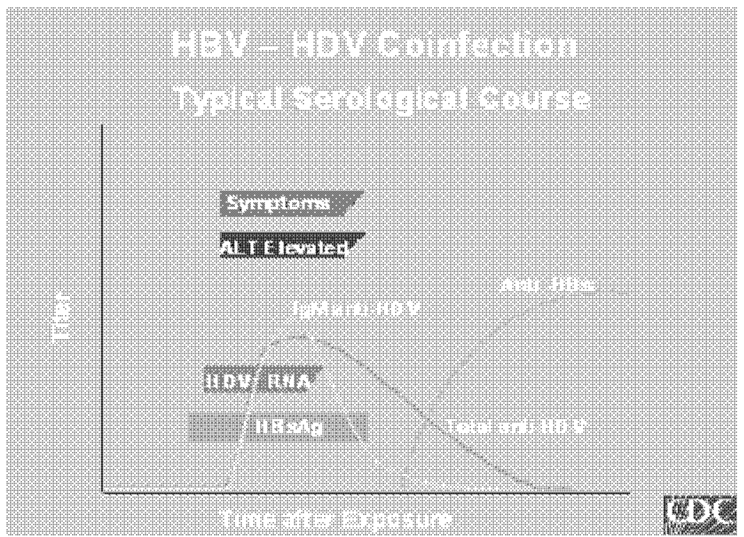
chronic liver diseases with cirrhosis compared with 15%-30% of patients with chronic HBV infection alone.

Hepatitis D Virus Modes of Transmission

- Percutanous exposures
  - injecting drug use
- Permucosal
  - exposures sex contact

Notes:

The modes of HDV transmission are similar to those for HBV, with percutaneous exposures the most efficient. Sexual transmission of HDV is less efficient than for HBV. Perinatal HDV transmission is rare.

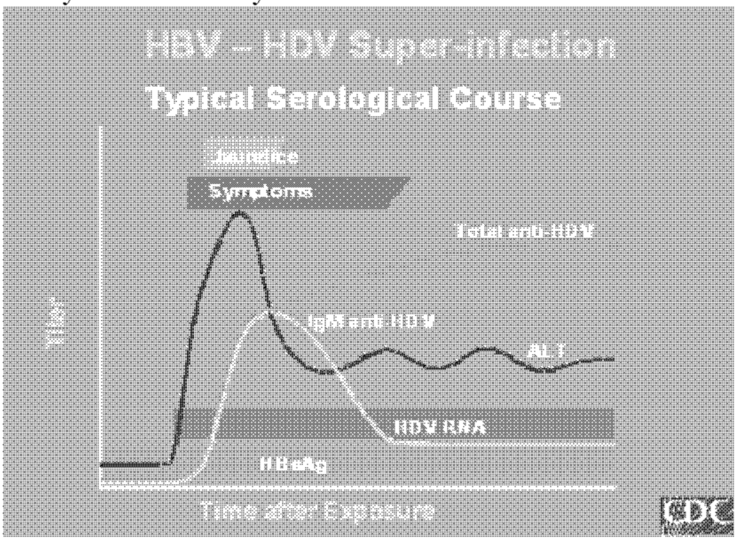


The serologic course of HDV infection varies depending on whether the virus is acquired as a co-infection with HBV or as a superinfection of a person with chronic HBV infection.

In most persons with HBV-HDV co-infection, both IgM antibody to HDV (anti-HDV) and IgG anti-HDV are detectable during the course of infection. However, in about 15% of patients the only evidence of HDV infection may be the detection of either IgM anti-HDV alone during the early acute period of illness or IgG anti-HDV alone during convalescence.

Anti-HDV generally declines to sub-detectable levels after the infection resolves and there is no serologic marker that persists to indicate that the patient was ever infected with HDV. Hepatitis Delta antigen (HDAg) can be detected in serum in only about 25% of patients with HBV-HDV co-infection. When

HDAg is detectable it generally disappears as HBsAg disappears and most patients do not develop chronic infection. Tests for IgG anti-HDV are commercially available in the United States. Tests for IgM anti-HDV, HDAg and HDV RNA by PCR are only available in research laboratories.



In patients with chronic HBV infection who are super-infected with HDV several characteristic serologic features generally occur, including: 1) the titer of HBsAg declines at the time HDAg appears in the serum, 2) HDAg and HDV RNA remain detectable in the serum because chronic HDV infection generally occurs in most patients with HDV superinfection, unlike the case with co-infection, 3) high titers of both IgM and IgG anti-HDV are detectable, which persist indefinitely.

Hepatitis E

Recently identified cause of enteric ally transmitted non-A, non-B **(NANB)** hepatitis

Clinical Features

Incubation period 30-40 days
Acute, self limiting hepatitis, no chronic carrier state
Age: predominantly young adults, 15-40 years

Complications

Fulminant hepatitis in pregnant women. Mortality rate is high (up to 40%).
Virus cannot be cultured *in vitro*.

1) Calicivirus-like particles in the stool, by electron microscopy
2)Specific IgM in serum
3) PCR HEV-specific sequences in stool

HIV Infection

Characteristics

HIV is a retrovirus of the subfamily Lentivirinae. The virus contains an enveloped icosahedral helical capsid, which contains two identical RNA molecules. These RNA molecules are positive in polarity. The virus possesses a reverse transcriptase, and integrase, as well as other proteins.

After entering the cell by membrane fusion the virus RNA is transcribed to a DNA copy. This DNA migrates to the nucleus where it is integrated into the host chromosome via the viral integrase enzyme. There are two distinct phases of transcription. In the early phase, RNA strands or transcripts produced in the cell's nucleus are snipped into multiple copies of shorter sequences by cellular splicing enzymes. When these reach the cytoplasm, they are only about 2,000 nucleotides in length. These early transcripts encode the virus's regulatory proteins. In the second phase of transcription, two new size classes of RNA (long and medium [singly spliced]) are made and go to the cytoplasm. The medium transcripts encode the structural proteins. The long transcripts (full size) become the encapsidated genome. HIV encodes nine genes that are put into three classes, 1) Gag, Pol, and Env – the 3 major structural proteins, 2) Tat, and Rev – two regulatory proteins, and 3) Nef, Vif, Vpu, and Vpr – four accessory proteins.

Pathophysiology

HIV primarily infects CD4+ T cells (i.e., T cells that have a CD4+ receptor). To some extent monocytes and macrophages can also be infected.

HIV binds to the CD4+ receptor and subsequently to the CCKR5 coreceptor (whose normal function is a chemokine receptor). It then enters the cell by fusion with the plasma membrane. It uncoats and the reverse transcriptase makes a DNA copy, which is subsequently used as a template to make a double-stranded DNA.

One to three weeks after infection most patients exhibit a brief glandular fever-like illness, which is associated with a high titer of virus in the blood and a decline in CD4+ T cells. A vigorous cellular and humoral immune response commences and within a month or so the viremia declines to near undetectable levels.

CD8+ cytotoxic T cells, natural killer cells and antibody-dependent cell mediated cytotoxicity may all contribute to this decline by lysing infected cells, while neutralizing antibodies may mop up free virions.

After the initial phase, there is a long asymptomatic period of clinical latency, lasting anywhere from 1 to 15 years. During this period, only low titers of virus are demonstrable in blood and only a small number of circulating T cells are infected. However, ten times higher levels are detected in lymph nodes. As time passes there is a steady decline in CD4+ T cells. When the T cell count falls below 200-400/ul, opportunistic infections with various microorganisms may occur, and eventually the depleted immune system is unable to cope.

Activation of CD4+ T cells renders them permissive for HIV replication, and other viruses such as HHV-6, CMV, HTLV, and hepatitis B virus. Death is a result of these and other infections, malignancy, or a cachexia-like state. The average asymptomatic period is 11 years.

One of the more important questions about HIV is why does it take so long to cause the disease. After all, within a few weeks of infection there can be a high viral titer in the blood. The answer, it turns out, it quite complex and not well understood.

Many scientists think that the progression of AIDS is a monumental struggle between the AIDS virus and the immune system. In the beginning the immune system is primed and reduces the virus to negligible levels. However, it is not able to totally eradicate the virus because HIV has the advantage of being able to enter the proviral state and exist as nothing more than DNA inside a cell. It does not replicate in resting T cells and prefers to sequester itself in the lymph notes and not show itself in the bloodstream. Because of this, it can exist for long periods of time. It also has the advantage of being able to move from one cell to another by syncytia formation, thereby evading the immune system. Over a period of time, the virus slowly wins the race and defeats the immune system by destruction of T cells. This is quite noticeable in lymph nodes, which can be nothing but ravaged hulks by the end of the disease.

The mode of destruction of T cells is not exactly known, but there are many theories. They include:

1. the virus may leave some of its spike attached to the CD4+ receptor, thereby impairing T cell function.
2. syncytia formation
3. HIV may act as a super antigen, which has the effect of depleting T-cell populations.
4. HIV may trigger apoptosis

5. HIV may kill the cell by replication
6. HIV may trick the immune system to kill its own T4 cells (autoimmunity)
7. Cofactors (such as HHV-6) may aid in T cell killing

One of the more interesting AIDS episodes occurred when two gay men came to the NIH and told the researchers that they had had frequent and unprotected sex, yet they were still HIV-negative. They offered themselves as research models. Scientists determined that these men had a mutation in the CCKR5 coreceptor gene (R-5). Therefore, HIV could not easily enter their T cells.

Method of Transmission:

The accepted modes of spread are by:

- Sexual intercourse
- Direct injection with HIV-contaminated drugs-needles, etc, and
- From HIV-infected mother to fetus in utero, during childbirth, from mother to infant, or during breast-feeding.

Acquired Immunodeficiency Syndrome (AIDS)

Syndrome with the following features:

1) **Constitutional disease:** fever, diarrhoea, weight loss, skin rashes
2) **Neurological disease**: dementia, myelopathy, peripheral neuropathy
3) **Immunodeficiency:** Increased susceptibility to opportunistic infections: (see table 1)
4) **Rare malignancies**: Kaposi sarcoma, oral hairy leukoplakia, lymphomas.

Kaposi sarcoma - is a tumour of endothelial cells. Prior to the AIDS epidemic, this tumuor was rare and only found in middle aged African and Mediterranean Jewish men, in whom it was an indolent condition. AIDS patients develop a disseminated highly aggressive form of the disease.

Paediatric Infection

Following infection in the perinatal period, babies may develop a progressive illness in the first few months of life (No latent period). Clinical features include: Failure to thrive, diarrhoea, lymphadenopathy, susceptibility to opportunistic infections hepato-splenomegaly, lymphoid interstitial pneumonia and parotitis.

LABORATORY DIAGNOSIS

Serology

IgG develops 4-6 weeks post exposure and remains detectable for life. Its presence in serum therefore indicates infection.

Exception: Uninfected infants of HIV positive mothers

Direct detection of virus

-p24 antigen detectin by ELISA

-culture from peripheral blood lymphocytes (PBMC's)

-PCR

VACCINE PROSPECTS

There is no effective vaccine available for HIV. Attempts have been made to develop a vaccine, using:

- purified viral envelope glycoproteins, gp120 or 160
- whole inactivated virus
- live attenuated HIV strains (lacking certain genes)
- live recombinant virus vectors, expressing HIV proteins.

A major difficulty is the fact that neutralizing antibody in the serum does not protect the host from infection with HIV: Possible reasons for this include:

- Antibody enhancement of infection
- Rapid virus mutation may result in variation of envelope antigens (escape mutants)
- HIV can infect cells in sites that are sequestered from antibody
- Host may be infected by whole virus-infected cells

Anaerobic Infections

Definition of anaerobe:

It is a microbe that can only grow under anaerobic conditions only and is sensitive to metronidazole on routine disc testing anaerobically. Anaerobes are the most primitive bacteria in terms of oxidation (the greater the oxidation, the more advanced evolutionarily). They use a relatively inefficient metabolic system: **anaerobic fermentation** provides energy at 10% the level of aerobic respiration

To, prove that isolate is anaerobic bacteria

Subculture isolate both aerobically and anaerobically with CO2 enrichment for 5 days. Microaerophilic carbon dioxide dependent coccus appear on aerobic plate on fifth day, while strict anaerobe appear on 2^{nd} day under anaerobic conditions.

Classification of anaerobes:

Non Spore anaerobes (NSA):

Gram negative bacilli NSA

-Bacteroides fragilis.
-Prevotella melaninogemica
-Prophyromonas Spp.
-Leptotrichia buccalis(Vincentent s fusiform bacillus)

Gram negative cocci NSA.

-Fusiform species
-Veillonella species

Gram Positive Cocci NSA

-Anaerobic cocci
Peptococci
Peptostreptococci

Gram Positive Anaerobic Bacill NSA

-Propionobacteria anaerobic diphteroids skin common contaminants (a rare cause of infection after neurosurgery or heart valve operations)
-Actinomyces israelli anaerobic Gram positive branching bacillus the cause of actinomycosis).

Spore Forming Anaerobes:

CLOSTRIDIUM Species

-The anaerobic *Clostridium* species are large **anaerobic, Gram-positive, motile rods**. The *Clostridium* species are **spore-formers**, with spores generally wider than the diameter of the rod-shaped cells, and placement is central,

subterminal, or terminal. Most of the species are motile and possess numerous flagella all over the cell (**peritrichous flagella**).

-Many decompose proteins or form toxins, and some do both. The natural habitat of *Clostridium* species is soil or the intestinal tract of animals and humans, where they live as saprophytes.

- Clostridia produce serious disease including botulism, tetanus, gas gangrene, pseudomembranous ulcerative colitis, and food poisoning.

Clinically Important Colistridium Species:

-Clostridium tetani causing tetanus.

-Clostridium perferingens, Clostridium sporogenes, Clostridium oedemetiens causing gas gangrene.

- Clostridium Botulinium causing food poisoning botulinism.

-Clostridium difficile causing Pseudomembranous colitis.

Effect of Disinfectant on anaerobe

-Kill NSA, while spore forming anaerobes are resistant.

Predisposing Factors for anerobes infection:

Lower Oxygen tension and decrease oxidation-reduction potential.

1- Trauma with dead tissue in deep or extensive wound.

2- Impaired blood supply e.g. ischaemic arterial disease in foot or leg in diabetic patients.

3- Presence of other organisms- infection or colonization with other bacteria as E.coli in an abdominal wound may enhance growth of B.fragilis, or presence of synergistic infection as in Meleney's gangrene--- Staph aureus+ microaerophilic or anaerobic streptococci.

4- Presence of foreign bodies e.g clothes or soil inserted following car accident

<u>Autogonous Infection associated with NSA.</u>

-Mouth	Vincent s infection, gingivitis -necrotic purulent tonsillitis Dental sepsis may lead to anaerobic endocarditis	Leptotrichia, fusiforms and spirochetes Actinomyces israelli Anaerobic cocci and anerobic Gram negative bacilli
-Ear, Sinus	Chronic suppurative otitis media and sinusitis	Bacteroides species in a minority of cases
-Lower respiratory tract	Aspiration pneumonia Lung absess Empyema	Anaerobes from the mouth including Fusiform and anaerobic cocci occasionally Bacteroides fragilis
-Abdomen	Wound infection Abscesses or perotinitis associated with bowel cancer, appendicitis , diverticulitis and bowel surgery---bacteremia as complication	Faecal anaerobic bacteria mainly Bacteroides fragilis
-Skin, soft tissues	Infected diabetic ulcers, deep pressure sores, axillary abscesses, infected sebaceous cyst, Meleney s synergestic gangrene	Anaerobic cocci and anerobic gram negative bacilli
-Female genital tract	Post hysterectomy and occasionally post caesarean section wound infections Pelvic actinomycossis in association with intra-uterine device Pyosalpinx Septic abortion Bartholin s abscess	Fusiform, Prevotella Melaninenica , Bacteroides fragilis Anaerobic cocci Actinomyces israelii Fusiform, Prevotella melaningenica Bacteroides fragilis Anaerobic cocci

Spore Forming Anaerobes:

<u>1-Colistridium tetani</u>

-Gram+ rod with terminal spore drum stick appearance.

-Causes tetanus (lock jaw) in humans

-Spores can be acquired from any types of skin trauma involving an infected device
-If an anerobic environment is present, the spores will germinate and eventually form active C.tetani cells.
-At the tissue level, the bacterium releases an exotoxin that causes nervous system irregularities
-Toxin s effect includes constant skeletal muscle contraction
-Due to a blockage of inhibitory interneurons that regulate muscle contraction.
-Immunization prevents C.tetani infections in children and adults.
-The first four shots are administrated within two years of birth, followed with periodic booster shots given every ten years.

2-Colistridium perfringens:

-Can be contracted from dirt via large cuts or wounds
-Release of their exotoxin which causes necrosis of the surrounding tissue, also produce gas which leads to a bubbly deformation of infected tissues(gas gangrene).
-Can release an enterotoxin that may lead to severe diarrhea.

3-Colistridium botulinum:

-produce one of the most potent toxins.
-Causes the deadly botulism food poisoning.
-May find their way into foods that were placed in anaerobic storage such as cans or jars.
-Once the jars are sealed, the spores germinate and the bacteria release their potent toxin.
-Patient experience muscular paralysis and blurred vision.
-Immediate treatment with anti-toxin is required for the patient to survive.
-Infantile botulism is much milder than the adult version.
_Honey is the most coomon spores which germinate in the child s intestinal tract.
-Symptoms last a few days and then subside without the use of an antitoxin.

4-Clostridium difficile and Pseudomembranous Colitis

- Source

- feces of nondiarrheic humans: 5-10%
- hospital environment: up to 25% of patients
- soil, marine sediments (mostly spores)
- dogs and cats: up to 35%
- wide variety of other animals

- Disruption of intestinal flora by antibiotics, chemotherapeutics

- clindamycin: most cases per amount used
- ampicillin, cephalosporins: most commonly associated (more widely used)

- *C. difficile* often sensitive to antibiotics which initiate the episode
- Onset 4 - 10 days after start of antibiotic, up to 2 weeks after termination
- Transmission usually via spores, vegetative cells oxygen sensitive -> die rapidly

- aerosol (diarrhea), lack of hygiene (fecal-oral)
- pass through stomach
- bile acids induce germination.

- Antibiotic levels fall, *C. difficile* grows rapidly in unoccupied niches
- Vegetative cells produce toxins.

1-Toxin A 308 kDa (largest known exotoxin, maybe largest known prokaryotic protein).

-Enterotoxic *in vivo*: fluid accumulation <u>with</u> tissue damage (blood and mucus), cells can no longer control water movement

-Causes diarrhea, intense inflammatory response when fed to hamsters

-Chemoattractant for neutrophils

-Must be internalized for toxic effect

C-terminal 1/3:

- host cell binding (trisaccharide receptor)
- five repetitive peptides
- not toxic but required for toxic effect

N-terminal 1/3:

- toxin domain
- inactivation of Rho-protein by monoglucosylation (Rho-protein induces polymerization of actin)

Cytotoxic, cytotonic

2-Toxin B 269 kDa

-No enterotoxic activity *in vivo*

-Trace amounts of toxin A or mucosal damage necessary for toxic effect in rodent bowel

-cytotoxic *in vitro*(~1000-times more active than toxin A)

-63% amino acid homology with toxin A (gene duplication)

N-terminal domain: highly conserved, same activity as in toxin A

C-terminal domain: quite different, may recognize different receptor, also contains repetitive sequences

-Toxins act synergistically: toxin A damage to mucosal cells allows toxin B maximal effect
-Damage to colonic mucosa
- accumulation of fibrin, mucin, dead host cells (yellowish layer on surface = pseudomembrane)
- separate lesions coalesce
- Symptoms:
- severe abdominal pain
- water, non bloody diarrhea
- high number of neutrophils in stool
- Diagnosis:
- detection of the organism:
  * culture of feces for *C. difficile*(48-72h)
  * immunological assay for somatic antigens
- detection of toxins:
  * tissue culture
  * immunological assay for toxin antigens

Treatment

- Fatality rate: 27-44% if untreated
- Treatment
- cessation of antibiotic, if possible
- treatment with anti-*C. difficile*-drugs: vancomycin, metronidazole
- extended course may be required to prevent recurrence
- restoration of normal intestinal flora: fecal enema from family member
- Prophylaxis
- feeding of *Saccharomyces boulardii* (nonpathogenic yeast)
- administration of toxin-neutralizing antibodies

Microbiological diagnosis of the anaerobic Infections:

Proper management of anaerobic infections depend on appropriate documentation of the bacteria causing the infection, as certain or all anaerobes may not recovered when the specimen is not placed under anaerobic condition.

1-Selection of specimens for anaerobic culture:

Anaerobic infection can occur in all body sites including CNS, head and neck, oral cavity, chest, abdomen, pelvic, urogenital, skin and soft tissues and blood. So, the specimens that are suitable for isolation of anaerobes are
-----Blood samples
-----Aspirated pus
-----Aspirated body fluids ----CSF

Pleural fluid
Peritoneal fluid
Synovial fluid
Pericardial fluid
-----Direct lung aspirates, trans- tracheal aspirate, thoracocentesis or BAL.
------In urinary tract infections the suitable samples are by cystescopy or nephrestomy, ureterostomy or suprapubic aspiration.
-----Swabs are least advisable and less satisfactory than aspirates or pus samples.

2- Samples Transport;

Protection of anaerobic bacteria from oxygen exposure is critical step in the recovery of these organisms.

There are many of transport media:

1- The anaport system: Consists of 2 tubes with rubber stopper one contains sterile swab in oxygen free CO_2 or N_2 atmosphere and other contain few ml of reduced salt solution.
2- Vacutainer transport: used for swabs, fluid and tissue specimens. It consists of outer glass tube and inner glass and fixed with rubber stopper.
3- Biobag system: Clear gas impermeable bag commercially available.
4- Hungate tube, contains O_2 free gas and agar indicator system
5- Use of transport media Staurt's media or thioglycate broth in which the sample is immersed and covered and transported rapidly to the lab.

3-Laboratory Examination of anaerobic bacteria;

1-Direct examination
2-Culture and biochemical identification of anaerobes.
3- Antibiotic susceptibility tests
4-Serological Identification
5-Mollecular typing
6-Gas Liquid Chromatography

1-Direct Examination:
-Macroscopic
-Microscopic
-Macroscopic examination for foul smell, black colour, purulent appearance, necrotic tissues, gas or sulphur granules.
-Microscopic examination:
-Direct Gram Stain:---for the presence of cocci or bacilli in the sample
-Dark field examination or phase contrast for motile organisms or refractile spores.

2- Inoculation of Appropriate anaerobic media:
Non Selective media:
-Scheadler blood agar
-Brucella blood agar
-Columbia blood agar
-Brain heart infusion
-Trypicase Soya agar
-Egg Yolk agar
Selective Media
-Anaerobic Kanamycin-Vancomycin laked blood agar(AKVLB)
-Bacteroide bile esculin (BBE).
-Anaerobic Phenylethyel alcohol agar (PEA).
-Cycloserine Cefoxitin fructose agar(CCFA).
3-Anaerobic Incubation System.
The specimens are processed and incubated into appropriate media anaerobically at 37 C in any anaerobic systems
The anaerobic systems are:
1-Anaerobic jar.
2- Anaerobic work station or anaerobic chambers.
3-Anaerobic gas bags (biobag system).
Duration of anaerobic incubation:
At least 48 hours before examination of plate
-at least 72-96 hours for slowly growing suspected organisms.
-at least 9 days before discarding as negative sample.
Processing of colonies suspected to be anaerobes
Examination of each colony type appear in plate.
Colony morphology(shape, size, appearance, color, etc…)
Grome reaction
Pigmentation
Hemolysis
Flourescence
Biochemical Identification of the isolated colonies:
A-Preliminary
B-Definitive
A-Preliminary:
Colony morphology, Grom raction, pigments,hemolysis
Flourescence character
Specific disc—for peptostreptococus anaerobes

Nitrate disc for----B.ureolyticus and Veillonella sp.
Lipase test-----Clostridium spp, Bacteroides and Fusobacterium
Lecithinase---as above
Indole test for indole +ve give blue or green colour
Catalase test----For Bacteroides and Peptococcus
Bile test------ to differentiate bile resistant from bile tolerant
Urease test---for Clostridium and Bacteroid urelyticus
Ethanol spare test---- Clostidium species
Naglar test-----For Clostridium perferingens.

B) Definitive Identification of anaerobes:

1- Conventional tubed Biochemical Identification System;

Biochemically based this envolves preparing inoculum directely from pure culture and exposued to different tests as indole, sucrose, hippurate, etc…..
Commercially available systems include API20A, ATB32A, Minitik system.

2- Enzyme based systems : That based on the presence of performed enz. Consists of small plastic cards or panels that inoculated and require no anaerobic conditions. Most of them generate code number and referred to manufactures supplied code book.

Example---------ANI card of Vitek
----------- ANIDENT, rapid
------------Ana 11, Microscan walkaway
------------API Zym system

3-Antimicrobial Susceptibility of Anaerobes

1-Selection of an appropriate antimicrobial agents as:
- Metronidazole
-Clindamycine and Lincomycin
- Penicillin G
-Vancomycin for G+ve
-Macrolides –Erythromycine
- Zithromycin
-Carbapenem
-Cephalosporin (variable effect)

Method:

1-**Conventional Methods**

-Dilution method to asses MIC
Agar dilution Method
Broth Macrodilution method
Broth microdilution method

2-E test
3-Automated method as Vitek, sensititre and Walkaway
N.B Disc diffusion method is not suitable for anaerobes

4- Serological Identification

For Cl. Difficile and Cl. Botulinium toxins .
Flourescent antibodies for bacteroid spp. As fragilis, melaninog.

5-Molecular Identification:

To assess the pathogen genotype
We use----Restriction endonuclease analysis (REA)
----REA with pulsed gel electrophoresis
------Polyacrimide gel electrophoresis
-----------Restriction fragment length polymorphism (RFLP)
----------Hybridization methods
Ribotyping.

6-Gas Liquid Chromatography

To detect volatile free fatty acids of anaerobes (valeric acid,isovaleric acid, butyric acid and isobutyric acids-they are responsible for foul odour of anaerobeic infection) usefull for Bacteroides, Colistridium, and Fusebacterium spp.

Zoonoses

It is any infectious disease transmitted between humans and other vertebrate animals. The term was originally defined to describe a group of diseases that humans may acquire from domestic animals. This definition has been modified to include all human diseases that are acquired from or transmitted to any other vertebrate.

Mode of Transmission

-Contacts
-Inhalation
-Food and Milk products
-Excretions from animals e.g. saliva, stool, urine , blood and live tissues
-Arthropod vectors

Types of Organisms:

Bacterial:

Salmonella enteriditis
anthrax
Brucella
Mycobacterium tuberculosis
Campylobacter Vibrio
Leptospira
Listeria
Tularemia
Plague
Erysiploid
Glanders

Spirochate

Relapsing Fever
Lyme disease

Richetssiae

Q fever
Typhus

Chlamydia

Psittacosis

Viral

Rabies
Avian influenza

Fungal

Fungus species causing ring worm e.g. Trichosporum verrucosum

Parasite

Toxoplasma

Leishmaniasis

Laboratory Diagnosis

According to the organism suspected e.g.

- Dark ground microscopic examination for blood sample to detect Borrelia species causing relapsing fever.

-Blood film for Borrelia species stained with Geimsa and Leishman stains.

-Blood culture with extended duration for brucella, stool culture for Sallmonella enteriditis, culture for Mycobacterium tuberculosis, culture of scales from ring worm leision on Sabarouds media.

-Serological tests e.g. Brucella Widal test for Brucella, Weil Felix for Typhus, IgM for Toxoplasma.

-PCR for Brucella, avian influenza,

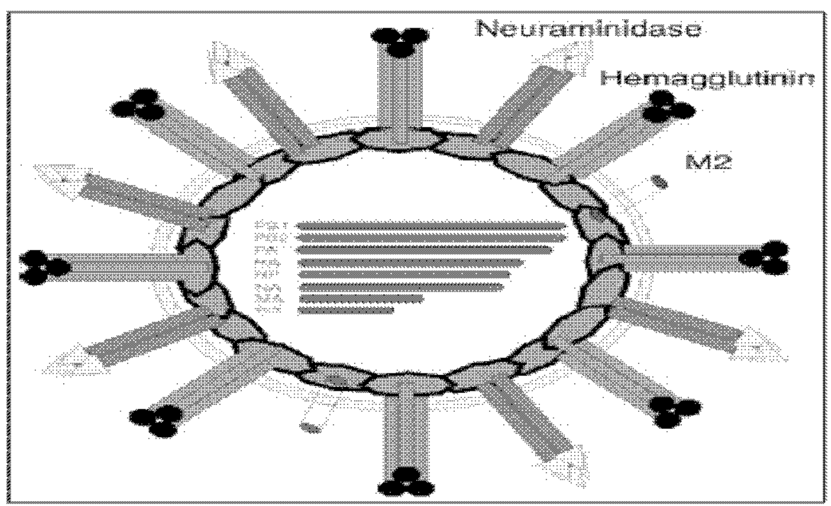


Figure 1—Schematic of avian influenza virus. Notice the 2 major surface glycoproteins, the hemagglutinin and neuraminidase, and the internal 8 gene segments (Graphic provided by D. Suarez, USDA, Athens, Georgia).

AVIAN INFLUENZA

Avian influenza virus (family: Orthomyxoviridae, genus: Influenza virus A) is a negative-sense, single stranded RNA virus with a genome composed of 8 gene segments that code for 10 proteins **(Fig 1)**.

The 2 surface glycoproteins, hemagglutinin and neuraminidase, are used for epidemiologic study of the virus. There are 15 hemagglutinin (H1 to H15) and 9 neuraminidase (N1 to N9) subtypes; however, the virulence of AI viruses for chickens does not correlate with these subtypes. Most infections with viruses (H1 to H15 subtypes) are subclinical or induce mild disease syndromes, whereas a few isolates of H5 and H7 subtypes induce severe, highly fatal disease, termed highly pathogenic AI (historically called fowl plague or fowl pest). The high virulence of H5 and H7 AI viruses in chickens does not correlate with their ability to infect and cause disease in humans.

Genetic Change in Influenza Viruses

Influenza viruses have the propensity to change genetically, which contributes to the interspecies transmission and zoonotic potential of AI viruses. Change can occur by
mechanisms: random mutations in the RNA genome, especially in the hemagglutinin, which occur gradually over time; and reassortment of the 8 gene segments that occurs abruptly between 2 influenza viruses that infect a single cell, resulting in progeny that are hybrid viruses. For human Influenza virus A, these phenomena have been termed drift and shift, respectively. The frequency of such occurrences varies with the infection rates in different animal species.

Reservoirs of Avian Influenza virus

In birds, the greatest diversity of influenza viruses (all combinations of the 15 hemagglutinin and 9 neuraminidase subtypes) is found in wild birds of the

orders (ducks and geese) and (shorebirds) and occasionally other aquatic bird species. I
nfections in these species are usually subclinical. These wild bird species serve as the primordial or primitive reservoir of all influenza A viruses and, over long periods, have been the source of influenza viral genes for all influenza A viruses of domestic poultry and mammals..

Diagnosis of avian Influenza in Human:

Sample:

Nasopharyngeal aspirates

Laboratory Diagnosis

-Virus isolation and identification in cell culture. but this delays obtaining a definitive diagnosis for 1 to 2 weeks.

-Immunoflourescent detection of antigen of avian Influenza in nasopharyngeal aspirate

-Recently, **polymerase chain reaction (PCR)** and **rapid real-time PCR (RRT-PCR)** tests have been developed and used in human and animal field diagnostic situations, respectively. The RRT-PCR was used in the 2002 low pathogenicity H7N2 outbreaks in Virginia poultry and New England live poultry markets.

Antimicrobials

Most microbiologist distinguish two groups of antibiotics agents used in the treatment of infectious disease

- **Antibiotics**, which are natural substances produced by certain groups of microorganisms
- **Chemotherapeutic agents**, which are chemically synthesized .
- A hybrid substance is a **semisynthetic antibiotic**, where in a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties
- Antibiotics may have a **cidal (killing) effect** or a **static (inhibitory (effect on a range of microbes.**
- The range of bacteria or other microorganisms that is affected by a certain antibiotic is expressed as its **spectrum of action**.
- Antibiotics effective against procaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be **broad spectrum**
- If effective mainly against Gram-positive or Gram-negative bacteria, they are **narrow spectrum**.

■ If effective against a single organism or disease, they are referred to as **limited spectrum**

A clinically-useful antibiotic should have as many of these characteristics as possible

-It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
-It should be nontoxic to the host and without undesirable side effects.
-It should be no allergenic to the host.
-It should not eliminate the normal flora of the host.
-It should be able to reach the part of the human body where the infection is occurring.
-It should be inexpensive and easy to produce.
-It should be chemically-stable (have a long shelf-life).
-Microbial resistance is uncommon and unlikely to develop .

Table of Common antibiotics and their Spectrum of activity

Chemical class	Examples	Biological source	Spectrum (effec against)
Beta-lactams (penicillins and cephalosporins)	Penicillin G, Cephalothin	Penicillium notatum and Cephalosporium species	Gram-positive bacteria
Semisynthetic penicillin	Ampicillin, Amoxycillin		Gram-positive a: Gram-negative bacteria
Clavulanic Acid	Clavamox is clavulanic acid plus amoxycillin	Streptomyces clavuligerus	Gram-positive a: Gram-negative bacteria
Monobactams	Aztreonam	Chromobacter violaceum	Gram-positive a: Gram-negative bacteria
Carboxypenems	Imipenem	Streptomyces cattleya	Gram-positive a: Gram-negative bacteria
Aminoglycosides	Streptomycin	Streptomyces griseus	Gram-positive a: Gram-negative bacteria
	Gentamicin	Micromonospora species	Gram-positive a: Gram-negative bacteria esp. Pseudomonas
Glycopeptides	Vancomycin	Streptomyces orientales	Gram-positive bacteria, esp. Staphylococcus aureus
Lincomycins	Clindamycin	Streptomyces lincolnensis	Gram-positive a: Gram-negative bacteria esp. anaerobic Bacteroides
Macrolides	Erythromycin	Streptomyces erythreus	Gram-positive bacteria, Gram-negative bacteria not enterics,

Macrolides	Erythromycin	Streptomyces erythreus	Gram-positive bacter Gram-negative bacte not enterics, Neisseri Legionella, Mycopla
Polypeptides	Polymyxin	Bacillus polymyxa	Gram-negative bacte
	Bacitracin	Bacillus subtilis	Gram-positive bacter
Rifamycins	Rifampicin	Streptomyces mediterranei	Gram-positive and Gram-negative bacte Mycobacterium tuberculosis
Tetracyclines	Tetracycline	Streptomyces species	Gram-positive and Gram-negative bacte Rickettsias
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacte Rickettsias Ehrlichia Borrelia
Chloramphenicol	Chloramphenicol	Streptomyces venezuelae	Gram-positive and Gram-negative bacte

<u>Microbial Resistance to Antibiotics</u>

1-The Genetic Basis of Bacterial Resistance to Antibiotics

Natural Resistance .Bacteria may be inherently resistant to an antibiotic .

- Gene that is responsible for resistance to its own antibiotic; or a

- Gram-negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic;
- Organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic.

-**Acquired Resistance** .Bacteria can develop resistance to antibiotics, e.g .

Acquired Resistance. Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously-sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria:

- - Mutation and selection (sometimes referred to as vertical evolution);
- - Exchange of genes between strains and species (sometimes called horizontal evolution). Some bacterial specieses are able to spread drug resistance to other strains and species during genetic exchange processes

Antifungal Drugs

- **Antifungal drugs are classified according to mode of actions into three groups**:

-Drugs acting on ergosterol on cell wall of fungus.

- Azole
- Terbinafine
- Amphotricine
- Polyene-Nystatin

-Drugs blocks protein and DNA synthesis

- -Flucytosine

-Drug acting on microtubules and spindle synthesis of DNA

- Grisoflavin

Chemotherapeutic agents for viral infections

- Agents that inactivate intact viruses (virucidal)
- Agents that inhibit viral replication at cellular level (antivirals)
- Agents that augment the host response to infection (immunomodulators

<u>Virucidal agents</u>

- May cause direct inactivation in a single step
- Can damage host cells as well as virus so use is limited
- Can be used in preventing transmission of viral infections
- Examples include
- detergents
- organic solvents
- **ultraviolet light**

<u>Antiviral agents</u>

■ Viral replication depends on host cell metabolic functions
■ Useful antiviral agents:
■ must inhibit virus-specific events
■ must not interfere with host metabolism which would result in toxicity to the host/person
■ Typically have a restricted spectrum of activity

1. **Site of action**

■ Aattachment to the host cell
■ Uncoating of the viral genome
■ Nucleic acid synthesis
■ Assembly of progeny virions
■ *Drugs inhibit ongoing replication at host cell level and*
■ Replication will resume on removal of drug
■ Agents are not effective in elimination of non-replicating or latent virus

Host Immune Responses and immunomodulators

■ Intact host immunologic response is essential for recovery from viral infections. I t can be used in the following situations؛
■ Immunosuppression due to cancer chemotherapy, transplantation or HIV infection are associated with higher rates of chronic viral infection (HBV)or reactivation (HSV)
■ Response to antiviral therapy may be delayed
■ Drug resistance viruses may be higher
■ Replace deficient host immune responses using
■ exogenous antibodies
■ interferon
■ augment cell-mediated immunity (CMI)

Antiviral drug resistance

■ Resistance results from mutations within the viral genome and the presence of selective drug pressure
■ Factors favouring emergence of resistance
■ high replicative load
■ high intrinsic mutation rate: RNA>DNA viruses
■ **degree of selective drug pressure (higher in prolonged or repeated courses of drug therapy**

Laboratory Methods of Antimicrobial Susceptibility Tests

■ **I-Phenotypic Method**
■ **II-Genotypic Method**

I-Phenotypic Method:

- **1-Disc diffusion tests**
- **Kirby – Bauer method**
- Tests with diffusion gradients of concentration . By this method the organism is seeded uniformly on the agar surface and exposed to a continuous concentration gradient of antibiotic diffusing from a paper disk (disk diffusion test)
- **Medium used :-**

• Must support good growth of the isolated bacteria e.g Muller – Hinton agar .

• Blood used in agar may interference with antibiotic activity which is highly bounded to protein.

- **Inoculum's preparation :-**

• **Diluted 5-10 colonies in sterile saline or nutrient broth .**

• **Colonies used depend on the number of organisms needed to produce semi-confluent growth e.g. – few numbers in rapidly growing bacteria e.g Klebsiella species than slowly growing species e.g enterococci .**

• ***Inoculation :-*** **The inoculums can be distributed evenly over the test plate by flooding the plate with the bacterial suspension and drying the surface while the plate in horizontal position .**

- **By sterile swab , squeezed the tube and run over the plate .**
- **By 100p from the suspension of the organism**
- **Number of disk** 6 in 8.5 cm diameter plate
- **Choose of antibiotic disks :-**

• **Members of antibiotics used for particular species by the preferred method.**

• **Infections at a particular body site.**

• **First line antibiotics that are commonly used are first tested .**

- **Isolated of species should not be tested with drugs that are valueless in therapy e.g. Gram positive not to be tested to polymyxin or aztroneam .**
- **One antibiotic representive of each group .**
- **Interpretation :-**
- **Measure the radial diameter around the disc not including the disk itself .**
- **Sensitive : Zone equal or larger to the sensitive zone of the control organism**

- Intermediated: the Zone is equal to reported intermediate which equal 3mm less than the control organism.
- Resistant the zone size of the test strain is smaller than 3mm of the test strain.
- **Limitation of disc diffusion tests-:**
- Not applied to slowly –growing, Fastidious organisms or anaerobes.
- -Mycobacterial and fungus susceptibility testing requires specific techniques
- - The reported sensitivity tests results not applied to clinical sites infections, e.g. –Salmonella Typhi to aminoglycosids.
- -Not related to the achieved serum levels or body fluid levels of antibiotics.
- **Sensitivity of isolated bacteria in vitro may not coordinate with activity in vivo:-**
- Drug is not adequately absorbed.
- The drug unable to penetrate in effective concentration into the least accessible site of multiplication of pathogen.
- Inactivation of drug by a concomitant drug resistant bacterium.

Resistant to drug may prove effective if:-

- Administered in high dose.
- Elimination of bacteria is helped by the immune system of the body.

Primary sensitivity tests:-

- In these tests the specimen serves as the inoculum's. When mixed well a portion of it is spread uniformly over part or whole of one or more plates and antibiotics discs are applied before the plates are incubated.
- Advantages:
- -Rapid results of susceptibility in the second day e.g day earlier than test on pure subculture.
- - In mixed culture, help to separate bacteria of different species with different susceptibility patterns.
- Help in rapid identification of bacteria with diagnostic susceptibility patterns e.g. MRSA.
- - Can be used to isolate pure organisms growth around certain antibiotics as the disc of antibiotic can act as selective media inhibiting the growth of certain bacteria e.g. yeasts around antibiotics discs.
- Disadvantages:
- -The primary inoculum's can not be measured.

- -The choice of antibiotics is difficult as the identity of the organisms is not known, so the choice will be suggestive on the organisms possible to be found.
- Uses:
- -Urine
- -Swabs from wounds or pus in emergency clinics.
- **-2-Dilution susceptibility tests:-**
- Micro-minimal inhibitory and minimal bactericidal activity methods.

How to choose MIC ?

- It is equal to or less than quarter or half the concentration of the antibiotic found in the infected tissues of the patient give the usual schedule of doses . Laboratory it the concentration of MIC measurement of antibiotics that inhibit the growth of isolated organism .
- MBC measure the concentration of antibiotic that prevent absolutely the bacterial culture .
- **Methods**
- **1. Broth dilution tests.** Serial, twofold dilutions of an antimicrobial ore incorporated into broth-containing tubes, which are then inoculated with standard number of organisms, usually l05—l06 colony-forming units (CFU) per milliliter. After the culture has been incubated at 35oC for 16—20 hours with traditional technology, the tubes are inspected for visible growth. (Rapid techniques are also available. See sec. E.) The MIC of the drug is the lowest concentration that prevents visible growth. If the tubes with no visible growth are subcultured quantitatively to a drug-free medium, the MBC of the antimicrobial can be determined.
- **Microdilution** susceptibility testing employs the same principles but uses wells on a microtiter tray rather than diludon tubes, permitting miniaturization and automation of the MIC determination
- **Agar** dilution **test.** The agar dilution test is very similar to the broth technique except that the antibiotic dilutions are incorporated into a solid medium and the inoculum, usually 104 CFU/ml, is applied as a spot to a small portion of the agar plate. The MIC again is recorded as the lowest antibiotic concentration that prevents visible growth. In contrast to the broth dilution technique, an MBC cannot be determined with agar dilution
- **Application:-**
- Serious infection where endpoint concentration is ended
- Disc diffusion yield inter mediate susceptibility

- Life threatening infection due to organisms with unpredictable susceptibility pattern.
- Fastidious or slowly growing organisms.
- Failure of antibiotic therapy
- Serious infections caused by organisms susceptible only to toxic agents
- Limitation
- Limitation
- -Difficult
- It needs the knowledge about the achievable level in serum or body fluid

Automated method

In the last several years, a variety of instrument-assisted identification and susceptibility test methods have been developed that permit generation of test results in a period of 6—9 hours. as opposed to the 15—24 hour time frame required with traditional overnight method. These newer ‘rapid” methods have, in general, been shown to provide test results nearly as accurate as those derived from traditional overnight tests, d0t the newer tests are more expensive. The clinical impact of this newer technology and whether it truly facilitates faster and more cost-effective patient care is undergoing clinical study. One study suggests the rapid tests have a positive impact on patient care. The exact role of rapid tests versus traditions’ awaits further clinical experience and comparative studies

4-Antimicrobial concentration gradient methods

The spiral gradient endpoint method employs an agar plate containing a continuous gradient of antibiotic concentration from the center of the plate to the edge; the test organism is applied to the plate in a radial streak, and the MIC is determined by measuring the distance of growth from the edge of the plate

-E test E test (AB Biodisk, Solnia. Sweden) is a method based on the diffusion of a continuous concentration gradient of an antimicrobial agent from a plastic strip into an agar medium. This newly developed in Vitro technique was created to overcome several of the disadvantages of the disc diffusion and dilution techniques and also to retain the principle of the agar dilution method by producing on accurate, reproducible, quantitative MIC result

Special Laboratory Situations for antimicrobial susceptibilities

1-Susceptibility testing of anaerobic bacteria

2- Determination of beta-lactamase production

3. Susceptibility testing of antibiotic combinations in vitro

-٤ Susceptibility testing of M. tuberculosis

-٥Fungal and viral susceptibility testing

2-Genotype –Based Detection Methods:

These methods include

-Simple and multiplex PCR

-Real-time PCR

-DNA sequencing

-Hybridization-based techniques, which include macro- and microarrays .

A common example for application of genotypes-based methods for antibiotics resistance is in the field of *Mycobacterium tuberculosis*. Resistance in *Mycobacterium tuberculosis* arises via chromosomal mutations and is another major public health issue for which molecular tests are already available. These include, for example، a line probe assay for detecting mutations responsible for rifampicin resistance.

Monitoring Antimicrobial Therapy

Two general types of in vitro tests are used to monitor antimicrobial therapy: measurement of blood or body fluid antibiotic activity against the responsible organism, and assay of actual antibiotic concentrations in blood or other bodily fluids.

I. The serum bactericidal test determines the killing power" **of patient serum** against the infecting organism. The result is expressed as the highest dilution of serum that will produce the desired effect.

A. Method. The serum bactericidal test involves a modification of the broth dilution technique. Serum usually is obtained from the patient at times believed to correlate with the maximum or minimum antibacterial activity. Serial, twofold **dilutions of the patient's serum are inoculated with a standard quantity of the infecting organism.** After overnight incubation of the mixtures, the inhibitory and lethal end points are determined as in broth dilution susceptibility testing

The serum inhibitory or bacteriostatic activity is defined as the highest dilution of serum that demonstrates a visible inhibitory effect. **The serum lethal or bactericidal activity** is similarly expressed as the highest dilution that produces a lethal effect, usually defined as a 99.9% or greater reduction of viable organisms in the initial inoculum. Bactericidal activity of other bodily fluids such as CSF, urine, and synovial fluid also can he measured by a modification of this method.

1. **Variables.** The measurement of serum bactericidal activity is influenced by numerous technical variables. These include the type of serum or broth diluents used, whether serum complement is or is not inactivated, the concentration of magnesium and calcium ions in the media, and the definition of the bactericidal end point. For example, a patient's serum containing highly protein-bound antibiotics may show greater bactericidal activity if diluted in nutrient broth (which has a low protcin concentration) rather than in pooled human serum (which has a high protein concentration). The **lack** of **interlaboratory standardization in the performance of these tests makes it difficult to compare results between studies** (43, 43a].
2. **Timing of sample.** Several authors favor collection of the serum sample at peak, whereas others prefer trough levels. The utility *of* peak versus trough serum bactericidal activity remains controversial [431.

B. **Clinical application.** Many authorities advocate using serum inhibitory or bactericidal activity as the best indicator of potential therapeutic efficacy. The test is the most reliable in vitro correlate of actual in vivo conditions because it

accounts for other components of the antibacterial activity of serum in addition to the antibiotic (i.e., serum complement, opsonins, lysozymes).
However, clinical applicability of the serum bactericidal titer remains to be proven rigorously, and its use remains somewhat controversial. **infectious disease consultation is advised to assist in the appropriate utilization and interpretation of serum bactericidal tests.**

A determination of serum bactericidal activity may prove useful in guiding therapy, particularly in the following situations:
1. Endocarditis may be more effectively treated when higher serum bactericidal activity can be achieved. However, the results of serum bactericidal tests are not necessarily predictive of survival or clinical cure, and the peak and trough bactericidal titers that best correlate with outcome are not yet clear [43, 43a, 451. Although a peak bactericidal titer of at least 1:8 is most frequently recommended, one study concluded that a peak titer of 1:64 or more and a trough titer of 1:32 or more were most predictive of bacteriologic cure of endocarditis; the test was a poor predictor of bacteriologic failure [46].
The bactericidal titer may be particularly helpful in the following circumstances:
a. When endocarditis is caused by organisms that are not highly sensitive to the antibiotics being used, and a synergistic combination of antibiotics might be more effective
b. When less well established treatment regimens are employed
c. When the patient fails to improve on standard therapy
d. When the serum bactericidal titer is very high and drug toxicity is a significant risk, in which case the drug dose might be reduced without compromising antibacterial effect
2. In acute and chronic **osteomyelitis,** serum bactericidal titers that exceed certain levels have been correlated with cure [47]. When changing from parenteral to oral therapy of acute hematogenous osteomyelitis in children, bactericidal titers often are monitored to adjust antibiotic dosage to achieve a bactericidal level of 1:8 or more [43a]. The usefulness of serum bactericidal tests in the management of osteomyelitis, particularly in adults, remains uncertain.
3. In the **immunocompromised host, a** serum bactericidal titer of 1:8 or greater has been correlated with successful treatment of bacteremia and soft-tissue infections [48]. Higher bactericidal titers may be desirable in the granulocytopenic patient with gram-negative rod bacteremia [49].

4.In patients with acute pulmonary exacerbations of **cystic fibrosis,** peak serum bactericidal titers of 1:128 or greater against the patients' pulmonary pathogens have been correlated with favorable bacteriologic responses to therapy [50].

II.Antimicrobial levels may be obtained to assess the adequacy of the chosen dose and route of administration and to avoid toxicity [19].

A. Methods

1. Correct timing of samples is necessary for accurate interpretation of the significance of antibiotic levels. The two measurements usually performed are the anticipated peak and trough blood levels of the antimicrobial after a dose has been given.

a. Peak blood levels usually **are** obtained 1 hour after an intramuscular dose, 30 minutes after the completion of an intravenous infusion, or 1—2 hours after an oral dose. In patients with renal insufficiency who receive antimicrobials by the parenteral route, peak levels may be delayed *2—4* hours after an intramuscular antibiotic dose or 1 hour after an intravenous dose.

b. **Trough blood levels** are obtained immediately before the next dose is due.

c.The blood should be obtained in tubes free of anticoagulant.

d. The sample should be taken promptly to the laboratory and quickly processed. Some antibiotics rapidly lose activity, and the simultaneous piesence of two antibiotics may result in one agent inactivating the other (e.g., carbenicillin can inactivate gentamicin).

e.The laboratory **requisition** should indicate clearly the antibiotic level desired, time of most recent dose, amount of most recent dose, and any concomitant treatment with other antibiotics.

2. **Techniques for assay of antibiotic levels.** Prior **to** 1970, bioassays (agar diffusion and broth dilution) were the most commonly used techniques to assay for levels of antibiotics in bodily fluids. 13ioassnys have been largely supplanted by a variety of more accurate and reproducible methods (e.g., immunoassays and high-pressure liquid chromatography),

a.**Bioassays** are performed by parallel dilution of both antibiotic standards and the patient's bodily fluid. The dilutions then are tested for their ability to inhibit the growth of an indicator organism. The quantity of antibiotic in the bodily fluid is derived from the relationship between the degree of inhibition of the indicator organism by the bodily fluid and the inhibition by the antibiotic standards. Because bioassays depend on the inhibitory effects of an antibiotic on an organism, they lack specificity (i.e., they cannot differentiate between the effects of two or more antibiotics present in a bodily fluid). Therefore, it **is essential to submit complete and accurate information about combination**

antimicrobial therapy with specimens sent for bioassay. With such information, the laboratory can sometimes circumvent the problem by technical manipulations (e.g., add beta-lactamase to inhibit penicillias, use niultidrug-resistsnt indicator organisms, or remove antibiotics with cation-exchange resins). **Most bioassay systems are not as precise as other types of assays but, when** the tests are performed carefully with adequate controls, the precision generally is adequate for clinical use.

b. Immunologic assays are presently the most **widely used** method for de-termining antibiotic levels in bodily fluids. They exploit the specificity of the antigen-antibody (antimicrobial-antibody) reaction and use sophisticated instrumentation. More simple latex agglutination tests have also been developed and marketed for the semiquantitative assay of aminoglycoside antibiotics. Immunoassays have gained widespread acceptance because they are rapid, accurate, specific, and easier to perform than bioassays. **Aminoglycoside** and **vancomycin** levels now are routinely available in many laboratories using the immunoassay method.

c.**High-pressure liquid chromatography** is a method for separating com-pounds; quantitation is subsequently achieved by analysis of the separated compounds. Liquid chromatographic procedures have been developed to measure almost all antibiotics in clinical specimens but are used most widely for chloramphenicol because no suitable immunoassay has been developed for this drug. Immunoassays generally are favored because they are simpler to perform.

B.Clinical application. Determination of antibiotic levels may be considered in the following situations:

1. When complicated or life-threatening infections exist secondary to organisms with MIC or MBC values near the maximum achievable levels of the antibiotic being used. Pneumonia and bacteremia due to gram-negative organisms may respond more favorably to treatment when therapeutic plasma levels of aminoglycosides are achieved. A high peak concentration of aminoglycoside relative to the MIC for the infecting organism has been correlated with improved clinical response to therapy.
2. When one wishes to monitor therapy with an antibiotic that could have toxic side effects, particularly in the presence of altered hepatic or renal function (e.g., aminoglycosides).
3. When an infection due to a sensitive organism is not responding to antibiotic treatment and all other therapeutic approaches have been optimized.

C. Interpretation. As a general guide, it is anticipated that an infection will respond to therapy if a level of antibiotic greater than the MIC of the infecting organism can be achieved at the site of infection. However, the relationship between achievable serum levels and response at an extravascular site of infection is variable. Also, factors other than an absolute serum level may be important (e.g., magnitude of level in comparison to MIC, duration of level above the MIC, and effect of serum protein binding). **Determination of an antibiotic level is not a substitute for clinical judgment,** and other therapeutic modalities must always be optimized (e.g., draining abscesses, removing foreign bodies, and bolstering host defense mechanisms).

Biosafety Measures in the Clinical Laboratory

- **Basic laboratories, containment laboratories, and maximum containment laboratories**
- **Biologic safety cabinets**

Clinical laboratories are special, often unique, work environments that may pose identifiable infectious disease risks to persons in or near them. These infections have been recognized for many years. In a series of published early surveys, Pike and associates[1-4] reported over 3,000 cases of laboratory-acquired infections, including brucellosis, tuberculosis, typhoid, streptococcal infections, and hepatitis. These incidents, along with considerable anecdotal information, suggest that most laboratory-acquired infections occur as a result of error, accident, or carelessness in the handling of a known pathogen; often the mode of transmission is unknown.

During the 1970s, in an effort to reduce the risks of infection in the laboratory, scientists devised a system for categorizing etiologic agents into groups based on the mode of transmission, type and seriousness of illness resulting from infection, availability of treatment (eg, antimicrobial drugs), and availability of prevention measures (eg, vaccination). The etiologic agent groupings were the basis for the development of guidelines for appropriate facilities, containment equipment, procedures, and work practices to be used by laboratorians. These guidelines, now referred to as biosafety levels 1 through 4, are published and regularly reviewed by the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH).

Biosafety level guidelines recognize that facility design is important in providing a barrier to protect persons working in the facility as well as those in the community. An accidental release of certain airborne infectious agents could be catastrophic. To assist in planning and managing a laboratory, the CDC describes 3 facility designs based on functions in handling infectious agents.

Basic Laboratory

The first design, known as the basic laboratory, provides general space in which work is done with viable biosafety level 1 agents (eg, *Bacillus subtilis, Naegleria gruberi*), which are not associated with disease in healthy adults, and biosafety level 2 agents (eg, hepatitis B, salmonellae), which pose minimal potential aerosol hazard to laboratory personnel and the environment. Basic laboratories include those that use biosafety levels 1 and 2. While work is commonly conducted on the open bench, certain operations are confined to biologic safety cabinets. Public areas and general offices to which non

laboratory staff requires frequent access should be separated from spaces that primarily support laboratory functions.

Biosafety level 2 used in the basic laboratory differs from biosafety level 1 in that:

1. Laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists;
2. Access to the laboratory is limited when work is being conducted;
3. Extreme precautions are taken with contaminated sharp items; and
4. Certain procedures in which infectious aerosols or splashes may be created are conducted in biologic safety cabinet or other physical containment equipment.

There is no specification for single-pass directional inward flow of air (a system in which air goes through the laboratory area once before being filtered) from a biosafety level 2 laboratory. However, because most microbiology laboratories also work with potentially hazardous chemicals, negative air pressure is usually present as well. There are published recommendations for preventing buildup of chemical vapors in laboratories, including use of chemical fume hoods and/or single-pass air when recirculation would increase the ambient concentration of hazardous materials.

Containment Laboratory

The containment laboratory has special engineering features that make it possible for laboratory personnel to handle aerosolized hazardous materials (eg, *Mycobacterium tuberculosis, Coxiella burnetii*, and St Louis encephalitis virus) without endangering themselves. More emphasis is placed on primary and secondary barriers to protect personnel in contiguous areas and the community from exposure to potentially infectious aerosols and to prevent contamination of the environment. This laboratory is usually described as a biosafety level 3 facilities.

The unique features that distinguish this laboratory from the basic laboratory are the provisions for access control and a specialized ventilation system. The containment laboratory may be an entire building or a single room (eg, for tuberculosis testing) in a basic laboratory. A containment laboratory is separated from other parts of the building by an anteroom with 2 sets of doors or by access through a basic laboratory area. Because of the potential for aerosol transmission, air movement is unidirectional into the laboratory (ie, from clean areas into the containment area), and all exhaust air is directed outside the building without any recirculation, or it undergoes high-efficiency particulate air (HEPA) filtration.

All procedures involving the manipulation of infectious materials are conducted within biologic safety cabinets or other physical containment devices. These facilities have solid floors and ceilings and sealed penetrations. They are designed and maintained to allow appropriate decontamination in the event of a significant spill. All waste from these laboratories must be rendered noninfectious before final disposal.

Maximum Containment Laboratory

The maximum containment laboratory has special engineering and containment features that allow activities associated with infectious agents (e.g., Lassa virus, Ebola virus) that are extremely hazardous to laboratory personnel or that may cause serious epidemic disease. This laboratory is considered a biosafety level 4 facilities. Although the maximum containment laboratory is usually a separate building, it can be constructed as an isolated area within a building. The laboratory's distinguishing characteristic is that is has secondary barriers to prevent hazardous materials from escaping into the environment. Such barriers include sealed openings into the laboratory, air locks or liquid disinfectant barriers, a clothing-change and shower room contiguous with the laboratory, a double-door autoclave, a biowaste treatment system, a separate ventilation system, and a treatment system to decontaminate exhaust air.

Within work areas of the facility, all activities are confined to class III biologic safety cabinets or class II biologic safety cabinets used by personnel wearing 1-piece positive-pressure body suits ventilated by a life-support system. Members of the laboratory staff have specific and thorough training in handling extremely hazardous infectious agents, and they understand the primary and secondary containment functions of the standard and special practices, the containment equipment, and the laboratory design characteristics. They are supervised by competent scientists who are trained and experienced in working with these agents.

All wastes are decontaminated before leaving the maximum containment laboratory, and the exhaust air is passed through HEPA filters. Except in extraordinary circumstances (eg, suspected hemorrhagic fever), the initial processing of clinical specimens and identification of isolates can be done safely at a lower level containment. The containment elements are consistent with the Occupational Safety and Health Administration Blood borne Pathogen Standard as well as those recommended by the National Committee for Clinical Laboratory Standards (M29-A).

Biologic Safety Cabinets

Various laboratory procedures generate aerosols that may spread biohazardous materials in the work area and pose a risk of infection to personnel. Biologic safety cabinets are used to prevent the escape of aerosols or droplets and to protect the research product from airborne contamination. These devices are distinct from horizontal or vertical laminar flow hoods, which should never be used for handling biohazardous, toxic, or sensitizing material. Chemical fume hoods also should not be used for biohazards as they are solely designed to protect the individual from exposure to chemicals and noxious gases. These chemical fume hoods are not equipped with HEPA filters. BSCs are designed to protect the individual and the environment from biologic agents and to protect the specimens and other materials from biologic contamination.

There are 3 general types of BSCs: class I, II, and III [**F1**] [**F2**] and [**F3**] There is 1 type of class I BSC. This cabinet is similar to a chemical fume hood with an inward airflow through the front opening. The exhaust air from the biologic safety cabinet is passed through a HEPA filter so that the equipment provides protection for the worker and the public. However, the specimens and other materials are potentially subject to contamination. Class I cabinets are not generally recommended for biohazard work.

Class II biologic safety cabinets are designed to protect personnel, the general public, and the specimen. The airflow velocity at the face of the work opening is at least 75 linear feet per minute (lfpm). Both the supply and the exhaust air are HEPA filtered. There are 4 types of class II cabinets (IIA, IIB1, IIB2, and IIB3). They differ in the amount of recirculation, downflow, and inflow. Usually, all but IIA are considered satisfactory for biohazard and toxic agents.

Class III cabinets are totally enclosed, ventilate cabinets of gas-tight construction, and offer the highest degree of protection from infectious aerosols. They also protect research materials from biologic contamination. Class III cabinets are most suitable for work with hazardous agents that require biosafety level 3 or 4 containment. All operations in the work area of the cabinet are performed through attached rubber gloves. The cabinets are operated under negative pressure. Supply air is HEPA filtered, and the cabinet exhaust air is filtered by 2 HEPA filters in series or HEPA filtration followed by incineration, before discharge outside of the facility.

Every day, new organisms are discovered that could potentially become pathogenic to the laboratory staff, patients, and visitors. It is up to the laboratory specialists in infection control, safety, and microbiology to recognize

these potential diseases and handle the organisms according to the NIH's most-recent biosafety guidelines.

Microbiology Laboratory Quality Control

General Requirements

For moderately and highly complex tests, the laboratory must:

- Follow the manufacturer's instructions.
- Have a procedure manual describing the process of the tests and reporting patient test results.
- Perform and document calibration procedures or check calibration at least once every six months.
- Perform and document control procedures using at least two levels of controls each day of testing.
- Perform and document applicable specialty and subspecialty control procedures.
- Perform and document remedial action taken when problems or errors are identified.
- Maintain records of all quality control activities for two years (five years for immunohematology).

Facilities

The laboratory must have space and environmental conditions necessary for conducting the services offered. This includes being constructed, arranged and maintained to ensure the space, ventilation and utilities necessary for conducting all phases of testing.

Safety precautions must be established, posted and observed to ensure protection from physical, chemical, biochemical and electrical hazards and biohazardous materials.

Test Methods, Equipment, Instruments, Reagents, Materials and Supplies

The laboratory must utilize test methods, equipment, instruments, reagents, materials and supplies that provide accurate and reliable test results and reports.

Requirements include

-Utilize appropriate and sufficient equipment, instruments, reagents, materials and supplies for the type and volume of testing performed and for the assurance of quality throughout the testing; and, test result reporting, including (as applicable): -Selecting methodologies and equipment and performing testing in a manner that provides test results within the laboratory's stated performance specifications for each method

-Water quality

-Temperature

-Humidity

-Protection of equipment and instrumentation from fluctuations and interruptions in electrical current that adversely affect results and reports

Document remedial actions implemented to correct conditions that fail to meet criteria

-Label reagents, solutions, culture media, control materials, calibration materials and other supplies including identification for:

Storage requirements-

-Identity, and when pertinent, titer, strength or concentration

-Preparation and expiration date Other appropriate information

-Prepare, store and handle reagents, solutions, culture media, control materials, calibration materials and other supplies in a manner to ensure:

- Items are not used when they have exceeded their expiration date, have deteriorated or are of sub-standard quality.

-Components of reagent kits of different lot numbers are not interchanged unless otherwise specified by the manufacturer

Control procedures

Remedial action to be taken when calibration or control results fail to meet the laboratory's criteria for acceptability.

-Limitations in methodologies, including interfering substances

Reference or normal ranges

-Imminent life-threatening laboratory results or panic (critical values. Must be informed at once by telephone to doctor e.g. Meningococci in direct gram of CSF.

-Pertinent literature references

Appropriate criteria for specimen storage and preservation to ensure specimen integrity until testing is completed.

The laboratory's system for reporting patient results including, when appropriate, the protocol for reporting panic values. Description of the steps to be taken in the event that a test system becomes inoperable. Criteria for the

referral of specimens including procedures for specimen submission and handling

Establishment and Verification of Method Performance Specifications

Prior to beginning to report patient results, the laboratory must establish and verify (for all tests and methods) performance specifications, including ----- Accuracy Precision/Analytical Sensitivity Analytical Specificity

-If applicable, the laboratory must also verify the reportable range of patient results as well as the reference (normal) range.

-When a new procedure is implemented, the laboratory must demonstrate, prior to reporting patient results, that it can obtain the performance specifications comparable to the manufacturer's established specifications.

-The laboratory must also verify the manufacturer's reference range for the laboratory's population. The laboratory must then establish calibration and quality control procedures based on the verified performance specifications. Each step must be documented in the laboratory's records.

Equipment Maintenance and Function Checks

The laboratory is required to perform equipment maintenance and function checks (electronic, mechanical and operational). These checks are considered to be necessary for proper test performance and result reporting to assure accurate and reliable test results and reports. The requirements for systems approved by the FDA include maintenance performance as specified by the manufacturer and with at least the frequency specified by the manufacturer. All maintenance performed by the laboratory should be documented. The same applies to system function checks - follow the manufacturer's instructions for both the checks and frequency and document each step

Calibration and Calibration Verification

The laboratory is required to substantiate the continued accuracy of each of its test methods, throughout its reportable range for patients, through calibration and calibration verifications

The reportable range of patient test results is the range of testresult values over which the laboratory can establish or verify the accuracy of the instrument, kit or test system measurement response.

Calibration is the process of testing and adjusting an instrument, kit or test system to provide a known relationship between the measurement response and the value of the substance that is being measured by the test procedure

Calibration verification is the assaying of calibration materials in the same manner as patient samples to confirm that the calibration of the instrument, kit

or test system has remained stable throughout the laboratory's reportable range for patient test results.

The manufacturer's instructions should be followed, along with using the materials specified by the manufacturer. Each step should be documented

Control Procedures

On a routine basis, the laboratory must perform control procedures to monitor the stability of the methods or systems utilized by the laboratory. Control and calibration materials indirectly assess the accuracy and precision of patient test results. At a minimum, the manufacturer's instructions are to be followed. The laboratory must:

Test quality control samples in the same fashion as patient specimens

Determine the statistical parameters (e.g., mean , standard deviation) for each lot number through repetitive testing. The stated values of an assayed control material may be used as the target values provided the stated values correspond to the methodology and instrumentation employed by the laboratory and are verified by the laboratory

Accept control results only when the laboratory's criteria for acceptability is met.

- Conduct reagent and supply checks for each shipment or batch of reagents, discs, stains, antiserum and identification system when opened. The checks should include positive and negative reactivity, as well as graded reactivity, if applicable. The laboratory must test staining material each day of use to ensure predicted staining characteristics
- For microbiology media, the laboratory must check for sterility, ability to support growth and, as applicable, selectivity/inhibition and/or biochemical response. The laboratory may use the manufacturer's control checks provided the manufacturer's checks meet the National Committee for Clinical Laboratory Standards (NCCLS) for media quality control.
- Document the physical characteristics of the media to confirm that the media has not been compromised. Report deterioration to the manufacturer.
- Follow the manufacturer's specifications for using the media.

Remedial Actions

- The laboratory must establish policies and procedures for remedial actions for quality control failures and apply them as needed to maintain accurate and reliable patient test results and reports. The laboratory must document when
- Test systems do not meet the established performance specifications. Examples include when equipment or methodologies perform outside

established parameters and when patient results are outside of the reportable range

- Control or calibration results fail to meet established criteria. When this occurs, patient results tested between the previous acceptable and the current unacceptable run must be evaluated to determine if the patient results had been affected. The laboratory must take remedial action to ensure the reporting of accurate and reliable results. The laboratory cannot report patient test results within its regular time frames. The laboratory must determine (based on the urgency of the tests requested) the need to notify the appropriate individual of the delay.
- Errors are detected in reported patient test results.
    - The laboratory must promptly notify the authorized person ordering the test or the individual utilizing the test results.
    - The laboratory must issue corrected reports promptly to the authorized person ordering the test or the individual utilizing the test results.
    - The laboratory must maintain exact duplicates of the original and corrected report for two years.

Specialty/Subspecialty Requirements

Along with meeting the general requirements, laboratories must also meet the following specialty/subspecialty requirements

Bacteriological laboratories Specific Precautions

The laboratory must check positive and negative reactivity with control organisms:

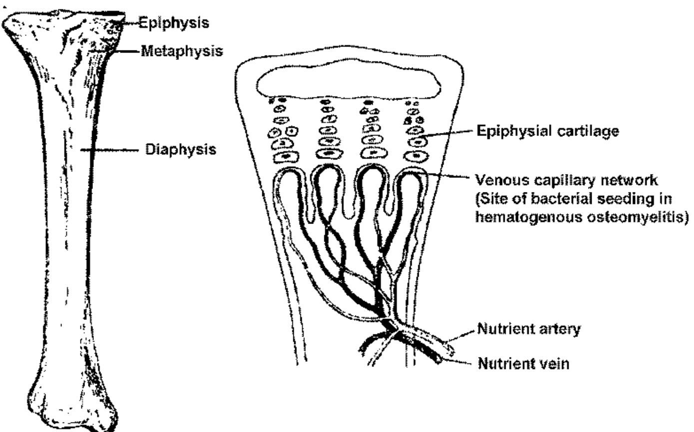
- Each day of use for catalase, coagulase, beta-lactamase and oxidase reagents and DNA probes.
- Each week of use for Gram and acid-fast stains, bacitracin, optochin, ONPG, X and V discs or strips.
- Each month of use for antisera.
- Each week of use for X V discs or strips (with a positive control organism).
- Each new batch of media and each lot of antimicrobial discs before or concurrent with the initial use, using approved reference organisms. Zone sizes or minimum inhibitory concentration for reference organisms must be within established limits prior to reporting patient results. Each day of use, test appropriate control organisms to check the procedure.

Skeletal Infections

The three basic types of skeletal infections are:

Osteomyelitis - infection of the bone
Septic arthritis - infection of the joint
Mycetoma - a local, chronic and progressive infectious disease of the skin, subcutaneous
tissues and bone

OSTEOMYELITIS



Long bone anatomy and vasculature

ETIOLOGY:

Osteomyelitis is a purulent inflammation of bone caused most often by bacteria and only occasionally by other microorganisms. In order of frequency of infection the bacterial species are:

Staphylococcus aureus)
Streptococcus spp.) Long bone and spine infections
Members of the Enterobacteriaceae)

Bacteroides spp. - mandibular infections

OVERVIEW:

Most osteomyelitis is of hematogenous origin. The clinical picture varies with age. In children through the age of puberty the long bones of the extremities are most often involved with the metaphysis as the initial infected site. In adults hematogenous osteomyelitis most often affects the spine. This age-dependent preference for bone relates to the vasculature and blood flow to the site. In children, the metaphysis is very active metabolic tissue with a large blood flow and with vasculature predisposed to infection. Phagocytes lining the capillaries in this region are deficient in number and function. The nutrient arteries near the epiphyseal cartilage are nonanastomosing, thereby allowing any blockage to produce tissue necrosis and the sinusoids (venous side of capillary) have slow, turbulent flow predisposing to thrombosis. As aging occurs metaphysis metabolism slows down, blood flow decreases and phagocytic activity increases. Concomitant with these changes the vertebrae become more vascular with maturation (senile hyperemia) and bacteremias seed vertebral bodies preferentially at the more vascular anterior vertebral end plates. In addition, lumbar paracentral veins communicate freely with pelvic veins by valveless anastomoses. Theoretically, retrograde flow from pelvic tissues (urethra, prostate, bladder) to lumbar vertebrae explains the spread of pelvic infections preferentially to lumbar vertebrae.

In about 25% of patients with osteomyelitis, the predisposing factor is trauma to the bone at or near the site of infection. Infections of the mandible are often due to traumatic dental procedures while the installation of prosthetic devices, such as artificial joints, predisposes to long bone infection.

PATHOLOGY:

The host responds to the presence of bacteria in the metaphysis with a local increase in vascular permeability, resulting in edema, increased vascularity and the influx of polymorphonuclear leukocytes. Pressure increases as pus collects

and is confined within rigid bone. Exudation through Volkmann's canals and the haversian canal affords little relief, although the relatively inelastic periosteum may become elevated. The blood supply to the area of involvement is decreased secondary to the pressure; necrosis of the infected bone may result in the formation of a sequestrum. A protein-rich liquid containing inflammatory cells may collect in an adjacent joint but such effusions are sterile.

After the vascular supply to the involved area has been interrupted and necrosis has occurred, the chronic phase of osteomyelitis is established. The residual dead bone acts as a foreign body, making the eradication of bacteria impossible until the sequestrum is removed.

If the infected area becomes well demarcated and the infection is contained, the acute inflammatory process may subside, leaving a subperiosteal accumulation of pus which may be discovered by tenderness on palpation. This relatively quiescent form of subperiosteal infection is termed a Brodie's abscess. After some time, there is deposition of new bone, the involucrum, under the elevated periosteum.

In osteomyelitis of the spine, infection most often involves the vertebral body. It spreads readily through the anastomotic venous system to adjacent ligaments and vertebral bodies. It is common for more than one vertebral body to be involved. Pus may accumulate between the vertebral periosteum and dura mater, forming an extradural abscess. Compression of the spinal cord may result, yielding a paraplegia. If a subdural abscess ruptures into the subarachnoid space, meningitis results.

CLINICAL SYMPTOMS:

Hematogenous osteomyelitis is often preceded by the signs and symptoms of bacteremia:

Fever	Inflammation
Malaise	Cephalgia
Myalgia	Anorexia

This phase of the illness may last for several days.

The second phase of the disease is the clinical onset of involvement of bone. This gives rise to:

Restricted motion
Pseudoparalysis
Soft tissue around the inflamed bone which is
Hyperemic
Warm
Edematous
Tender
Bone tenderness

DIAGNOSIS:

Diagnosis is based upon:

Clinical symptoms of an infection
Laboratory evidence of an infection:

Isolation of an organism
Increase in antibody titer

Presence of bone pain
Soft tissue swelling
Limited motion of extremity

Roentgenographic changes occur late in disease and should not be waited for to make the diagnosis; this would allow the development of chronic osteomyelitis. A differential diagnosis should include:

Rheumatic fever - there is severe pain and limitation of joint motion in this disease but there is no
bone tenderness.

Monoarthritic rheumatoid arthritis - the major swelling and tenderness is limited to the joint,
without local tenderness on palpation over the adjacent metaphysis.

Poliomyelitis - tenderness of the bone in an apparently paralyzed extremity indicates
osteomyelitis. There is no bone tenderness in polio.

Septic arthritis - joints are exquisitely tender and painful, whereas the swollen joint associated
with osteomyelitis may be gently manipulated through a limited range of motion.

Bacterial cellulitis - there is warmth, erythema, pain and edema of the soft tissue but it is clearly
demarcated whereas in osteomyelitis it is not clearly demarcated.

TREATMENT:

Acute osteomyelitis should be treated with a parenterally administered antibiotic based on the infecting organism:

Staphylococcus aureus - nafcillin
Streptococcus pyogenes - penicillin G
Gram-negative rods - ampicillin, gentamicin or chloramphenicol
Bacteroides spp. - clindamycin

Chronic osteomyelitis requires surgical procedures as well as antibiotic therapy. This includes full debridement and excision of all dead bone and necrotic tissue (sequestrectomy).

SEPTIC ARTHRITIS

ETIOLOGY:

Septic arthritis, the invasion of the synovial membrane by microorganisms, usually with extension into the joint space, is generally secondary to infection elsewhere in the body. In young adults, the primary infection is generally a genital lesion caused by *Neisseria gonorrhoeae*. In all other age groups the most common agent is *Staphylococcus aureus*, which spreads from a cutaneous lesion. Several other agents may cause septic arthritis but their frequency of infection is low.

OVERVIEW:

There is no microorganism that shows a tropism for synovial membrane and/or joints. During a septicemia, caused by an infection at a site outside of the joint, organisms are deposited in or on the synovial membrane and only rarely proliferate to cause a septic arthritis. When they do grow, the infection may spread to the joint space and then spread to bone and cartilage.

PATHOLOGY:

When joint infection occurs as a result of bacteremia, the initial growth of microorganisms is either in the synovial membrane or in the adjacent bone. In either case, an inflammation of the synovial membrane is quickly established and results in a marked increase in leukocytes in the synovial fluid, even though the fluid itself is sterile. When the microorganisms have spread into the joint fluid, culture of the fluid reveals the etiology of the infection. The pathologic findings are varied and depend on the duration of the infection, the organism and the resistance of the host. Early in the infection, only inflammatory changes in the synovium are seen. Late in the course of untreated septic arthritis, destruction of joint structures is marked. Articular cartilage is particularly vulnerable because it is an avascular tissue.

In acute, pyogenic arthritis, the cartilage characteristically dissolves first at points of articular contact to expose the underlying bone. As destructive changes occur several abnormalities appear in the synovial fluid:

Increased pressure
Low pH
Low concentration of glucose
Activation of proteolytic enzymes
Increased turbidity
Presence of mucin precipitate

CLINICAL SYMPTOMS:

The clinical manifestations of septic arthritis are variable and related to many factors: the etiologic agent, the joint involved and the age of the patient. In gonococcal arthritis one sees:

A prominent prodrome consisting of fever, chills, headache, anorexia and malaise
Migratory polyarthralgia or polyarthritis prior to localization in one or more joints
Skin lesions of gonococcemia
Small joint effusions
Tenosynovitis in about 1/3 of patients
Large joints are most involved

In nongonococcal septic arthritis the clinical picture is variable. At one extreme, the patient may complain of an acutely painful, swollen joint that is exquisitely tender and rigidly limited in range of motion but no manifestations of infection elsewhere. At the other extreme there may be little or no signs of inflammation.

DIAGNOSIS:

The definitive diagnosis of septic arthritis requires examination of the synovial fluid. This fluid will show:

Presence of microorganisms
Presence of antibody directed against the microorganisms
Turbidity
More than 10,000 pmns/mm^3
Decreased glucose concentration (< 0.6% of blood glucose)
Increased lactic acid concentration (> 65 mg/dl)

TREATMENT:

Treatment consists of both drainage of the synovial fluid and administration of an antibiotic systemically.

MYCETOMA

ETIOLOGY:

Mycetoma is caused by at least 20 species of actinomycetes and fungi. The most common infecting agents are *Nocardia* spp and *Madurella mycetomi*.

OVERVIEW:

Mycetoma is a local chronic and progressive infection of the skin, subcutaneous tissues and bone. It is characterized by swelling that is often grotesque and disfiguring and by multiple sinus tracts that drain granule-containing pus.

PATHOLOGY AND CLINICAL SYMPTOMS:

The disease is acquired by traumatic implantation in the skin. The microorganisms grow through the subcutaneous tissue into the bone. As this occurs, there is hyperplasia of the tissues, formation of pus containing granules (which are actually colonies of microorganisms), expression of pus to the surface of the skin and granuloma formation at the periphery of the infected area. The classical triad of symptoms is:

Gross deformity of the infected area
Draining sinuses
Granules in the pus

DIAGNOSIS AND TREATMENT:

Diagnosis is made by the presence of the classical triad of symptoms and by culture of the pus draining from the wound. Treatment is initially with sulfonamides for *Nocardia* or Amphotericin B for fungi. If the fungal infection does not respond to amphotericin B then amputation is required.

BACTERIAL SKIN INFECTIONS

I. Bacteria Skin Infections Are Common Problems in Dermatology and General Medicine

A. Appropriate diagnosis and treatment with appropriate presentation
B. Approach to recurrent and resistant infections
C. Review use of some of the new antibiotics
D. Stress importance of proper diagnosis culture and and underlying health status

II. Impetigo (Superficial epidermal bacterial infection)

A. Clinical characteristics

1. Golden-crusted erosions, superficial bullae or vesicles with turbid fluid
2. Expansion laterally in annular fashion
3. Usually on face, hands, genitalia, scalp
4. Pruritus possible
5. Typically, no accompanying constitutional symptoms
6. Ecthyma: deep impetigo often on legs, healing with scars

B. Etiology

1. Cause: cocci-type bacteria
   a. *Streptococcus*: usually non bullous
   b. *Staphylococcus*: commonly bullae and pustule

2. Poor hygiene, moist climates, crowding possibly exacerbating susceptibility

C. Associated conditions

1. *Staphylococcal* impetigo - possible association with immunodeficiency disease (e.g. chronic granulomatous disease of childhood)
2. Neonatal type - highly contagious and constant threat in nurseries
3. *STAPHYLOCOCCAL* SCALDED SKIN SYNDROME (SSSS) - Can be a truederm. emergency
   a. Generalized, confluent, superficially exfoliated. Dx occurring most commonly in neonates and young children.
   b. Must differentiate from TEN, which may be drug induced
   c. Caused by *Staphylococcus*, group 2-phage 71 - effect of exotoxin

4.. *Streptococcal* impetigo
   a. Post streptococcal glomerulonepohritis in 2-5% cases
   b. Glomerulonephritis (GLN): resolution in many cases but possible precursor of chronic renal disease (may not influence course, even if treated)
   c. Usually strains type 49, 55, 57, 60 and M-2 related to nephritis
5. Blistering Distal Dactylitis

a. Characteristic - tense, superficial blisters over tender erythematous base over volar fat pad of the phalanx
b. Age - usually 2 to 16
c. *Group A - beta streptococcus* as cause
d. Similar to *Strep. cellulitis*

D. Therapy
1. Removal of crusts
2. Topical antiseptics (Betadine) or antibiotics four times daily - mupirocin, bacitracin or polysporin; No neosporin (common topical sensitizer)
3. Systemic antibiotics
a. Pen VK - (drug of choice if *Strep.*) 250 mg QID
b. Erythromycin, cloxacillin or cephalosporin - PO 250-500 ig P.O. QID if *Staph*
4. Prevention of contact with other children (avoidance of same towels)

E. Recurrent *Staphylococcal* Impetigo
1. Topical Bacitracin or Mupirocin for chronic carriage (usually nasal)
2. Rifampin and Dicloxacillin
3. Rifampin and Trimethoprim
4. Bacterial Interferences - (*S. aureus* 502A)

F. Methicillin-resistant *Staphylococcus aureus*
1. Parenteral Vancomycin
2. Trimethoprim/sulfamethoxazole
3. Rifampin and Trimethoprim
4. Fluoroquinolones

III. Erythrasma
A. Clinical characteristics
1. Dry, scaly, reddish-brown plaques with the fine inconspicuous scale (looks like *T. cruris*)
2. Most commonly in axilla, groin, submammary areas

3. Frequently in obese, diabetic, or debilitated patients
4. Wood's lamp examination: coral red fluorescence visible

B. Etiology: *Corynebacterium minutissimum*

C. Differential diagnosis
1. Intertrigo: negative Wood's light examination
2. Dermatophytosis: positive KOH, active scaly border

D. Therapy
1. Cleansing, drying agents
2. Systemic erythromycin or tetracycline
3. Recurrence not infrequent

IV. Folliculitis - Furuncles - Carbuncles

A. Clinical Characteristics and Types
1. Superficial pustular follicular infection, usually *Staphylococcal* (Impetigo of Backhart).
2. Folliculitis
   a. Follicular pustules
   b. Pruritus very common
   c. Intertriginous areas commonly involved
   d. Exacerbation from moisture, poor hygiene
3. Sycosis Vulgaris
   a. Perifollicular beard area region presence of pustules and papules
   b. Pustules rupture after shaving and then from a fresh crop.
4. Furuncle (boil): deep infection with centra necrosis
5. Carbuncle
   a. Multiple boils with fistula formation
   b. Surrounding skin erythematous and tender
   c. Usual location: back, neck, intertriginous areas

B. Therapy
1. Folliculitis
   a. Removal of exacerbating factors
   b. Topical antiseptics, cleansing

c. Recurrent folliculitis: culture from patient's nose and contacts' noses; long
term antibiotics perhaps required

2. Carbuncles and furuncles
    a. Incision and drainage, lesion packed with gauze
    b. Systemic antibiotics after appropriate cultures
    c. If recurrent, search for underlying illnesses necessary: diabetes, leukemia,
    immunodeficiency disorders
3. Recurrent furunculosis, carbuncles and folliculitis
    a. Bacterial interferences
    b. Rifampin and dicloxacillin
    c. Clindamycin

V. Erysipelas (*Streptococcal* Cellulitis)

A. Clinical characteristics
1. Erythematous, swollen, tender, sharply marginated area with active advancing
borders
2. Induration common
3. Blisters over lesion possible
4. Acute course
5. Malaise, fever (usually greater than 100 F), leukocytosis, and lymphadenitis
6. Lymphedema: possible result from recurrent episodes

B. Etiology and pathogenesis
1. Beta-hemolytic *Streptococci*
2. Inoculation in skin, dissection along tissue planes
3. Occurrence more frequent in patients with chronic lymphedema, recurrent tendency

C. Therapy
1. Systemic penicillin, 1 gm daily for 10 days, or erythromycin
2. For recurrent infections prolonged therapy perhaps necessary

D. Resistant *Streptococcal* Cellulitis
1. Confirm organism is strept

2. Rifampin and Dicloxacillin
3. Prophylactic Benzathine - Pen.
4. Prophylactic erythromycin

VI. Cellulitis and Fascitis

A. Cellulitis

1. Characteristic suppurative inflammation involving particularly the subcutaneous tissue.
2. Causative agent - *Streptococcus pyogenes*, staph and others also implicated.
3. Usually some type of discernilele wound.
4. Often mild erythema, tenderness, malaise, chills and fever
5. Aggressive antibody therapy

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